ADAPTIVE EXPANSION OF BIODEGRADATION BY *PSEUDOMONAS PUTIDA* F1

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BY

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ABSTRACT

Pseudomonas putida F1 (*Pp*F1) catabolizes aromatic compounds via benzoate dioxygenase, phenylacetyl-CoA epoxidase, p-cymene-monooxygenase, and toluene dioxygenase-mediated pathways, and the latter is shown here to be highly flexible, supporting growth on previously untested aromatic alkenes, esters, amides, alcohols, amines, and multi-ring compounds.

P. putida F1 is a highly studied model aromatic hydrocarbon oxidizer that grows on relatively few mono-substituted benzenes despite its genome encoding a toluene dioxygenase enzyme that oxidizes more than 150 compounds. While toluene dioxygenase and toluene dihydrodiol dehydrogenase oxidize many mono-substituted benzene ring compounds, further enzymatic processing may be inhibited, leading to accumulation of the respective catechol or 2-hydroxy-6-oxo-2,4-dienoate intermediate. The present study demonstrated that *P. putida* F1 can undergo adaption leading to a more expanded growth range of mono-substituted benzene ring compounds than had been previously demonstrated. Studies with well-characterized mutant derivatives of *P. putida* F1 and growth on expected metabolites of the toluene dioxygenase pathway indicated that the newly described metabolism was dependent on the Tod pathway enzymes. This study also revealed the interplay between the Tod pathway enzymes and catabolism of the aromatic acids liberated by TodF. TodABCDEF processing of allylbenzene and 1-phenylethanol liberates 3-butenoic acid and lactic acid, respectively, both of which support growth of *P. putida* F1.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Aromatic hydrocarbons and bioremediation

Aromatic hydrocarbons (also called arene, aromatic compounds, or aryl hydrocarbons) are compounds that carry at least one benzene ring structure, which is a cyclic hydrocarbon with the chemical formula C_6H_6. Based on the benzene ring, benzene could have many different derivatives in terms of chemistry. It exists in nature, mainly produced by plants, however, it also can be found in petroleum related production areas or polluted places and coal (Fuchs *et al.* 2011).

BTEX (benzene, toluene, ethylbenzene and xylene) is the major component in crude oil and petrochemical products such as gasoline. Benzene, toluene and ethylbenzene are ranked as priority pollutants in the United States (USEPA priority pollutants, 1996). BTEX represents a major portion of the aromatic hydrocarbons in gasoline. In addition, BTEX is also suggested to be a carcinogen that causes life threatening problems in drinking water (Dean 1985; Snyder 2002; Sarma *et al.* 2011).

Nowadays, the problems of aromatic compounds that causes pollution are overwhelming and chaotic, which mainly caused by industrial processes. It

is very difficult to clean pollution with any physical or chemical ways efficiently. For this reason, bioremediation becomes not only an environmentally friendly, but also an economical and efficient approach for dealing with the contamination of aromatic compounds in soil and water (Dagley 1971; Tyagi *et al.* 2011).

1.2 Overview of aerobic biodegradation of aromatic hydrocarbon

Aromatic compounds are one of the prominent pollutants in the environment that cause problems (Fuchs *et al.* 2011). Fortunately, they are also common growth substrates for microorganisms in nature. This is to say, microorganisms have evolved to assimilate these aromatic compounds as carbon and energy sources. They mainly apply molecular oxygen to attack and break the stability of the resonance structure of the benzene ring with the help of oxygenases (Finette *et al.* 1984). For example, through oxidation, monooxygenase oxidizes benzene at the *ortho*, *meta* or *para* position of the ring, one at a time. In contrast, dioxygenases incorporate both oxygen atoms to the aromatic substrate to form *cis*-dihydrodiol intermediate products and then follow that by dehydrogenation to form a catechol (a dihydroxy benzene). Both monooygenase and dioxygenase pathways lead to breaking the benzene ring and metabolizing further to make intermediates that funnel into major cellular molecules (Fuchs *et al.* 2011).

There are two major ring-cleavage pathways, either the *ortho*-fission (extradiol) or the meta-fission (intradiol) cleavage pathway (Fuchs *et al.* 2011). Both pathways can lead to the end products for entering the TCA cycle and supporting the cell's growth as carbon and energy sources. The

benzene substrates used by the benzene/toluene dioxygenase pathway in *Pseudomonas putida* F1 is the main focus for this thesis (Figure 1).

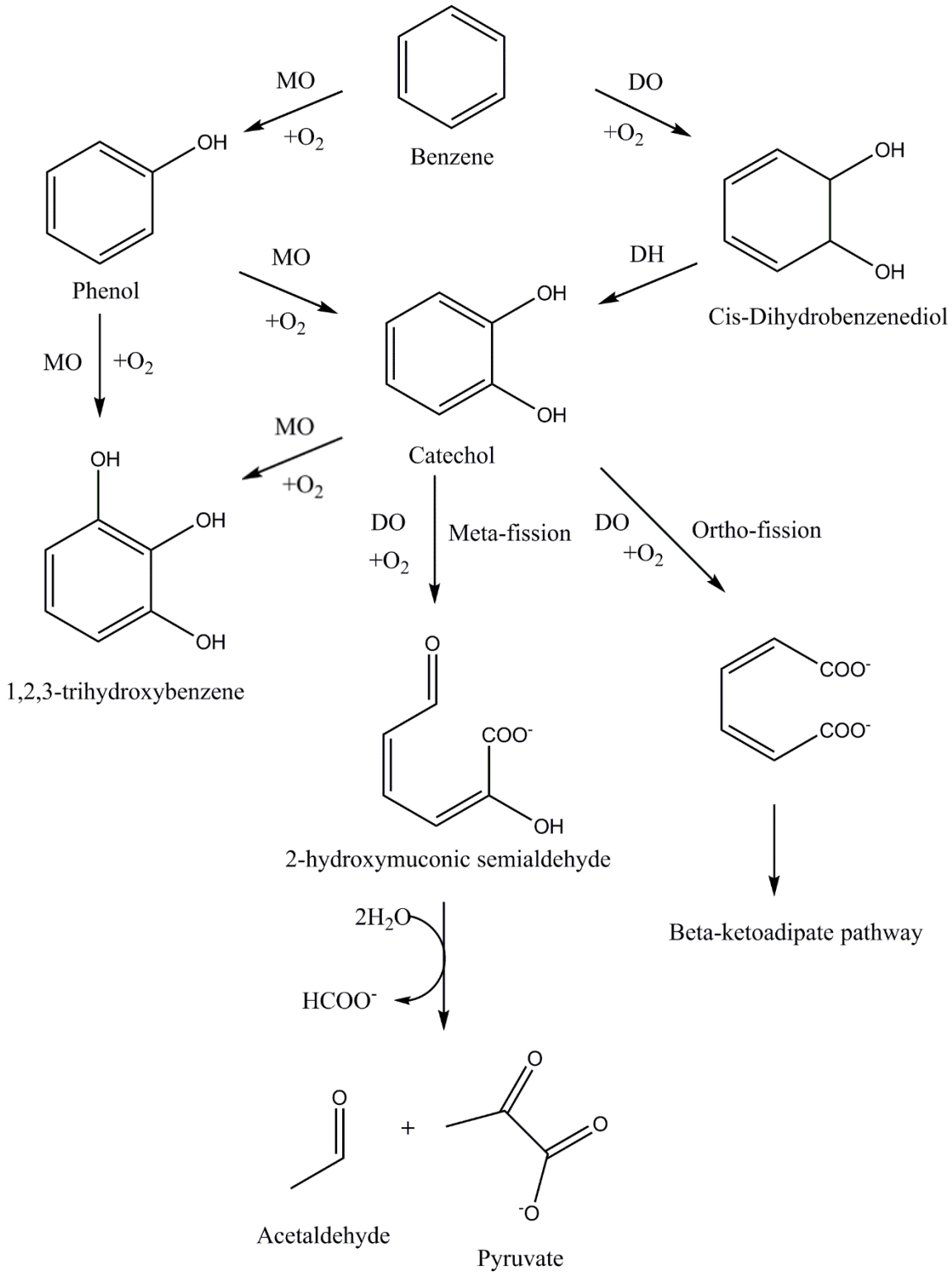

Figure 1. Oxygenase-initiated pathways for benzene rings showing ring cleavage pathway. Abbreviation: MO: monoxygenase; DO: dioxygenase; DH: dehydrogenase.

1.3 *Pseudomonas putida* F1

Pseudomonas putida F1 (*Pp*F1) is a gram negative, mesophilic bacteria with 6.2 million base pairs of DNA in its genome size. It is a highly-studied, model aromatic hydrocarbon oxidizer that grows on relatively few aromatic substrates despite its genome encoding a toluene dioxygenase enzyme that oxidizes more than 150 compounds. Over 200 publications have been published that involve *P. putida* F1. It was isolated from a toluene polluted creek in Urbana, Illinois. It was selected by its unique ability to grow with ethylbenzene as a sole carbon and energy source (Gibson *et al.* 1968a). The complete pathway and biochemistry for toluene dioxygenase-mediated biodegradation in *Pp*F1 has been well studied and characterized (Gibson *et al.* 1968a; Gibson *et al.* 1973; Gibson 1976; Yeh *et al.* 1977; Subramanian *et al.* 1981; Subramanian *et al.* 1985; Zylstra *et al.* 1988; Zylstra & Gibson 1989; Wackett 1990; Jiang *et al.* 1999).

*Pp*F1 is known to have several different pathways for oxidizing and assimilating aromatic compounds. *Pp*F1 is capable of catabolizing aromatic compounds via benzoate dioxygenase, phenylacetyl-CoA epoxidase, p-cymene monoxygenase, and toluene dioxygenase-mediated pathways. It has been studied for its narrow range of aromatic compounds that serve as growth substrates, identifying the metabolic pathways by which they are

metabolized, and investigating if these compounds are completely degraded and identifying intermediates and dead-end metabolites.

While toluene dioxygenase, has over 150 substrates, most of the oxidized compounds are not demonstrated as growth substrates for *P. putida* F1 (Gibson *et al.* 1968a; Gibson *et al.* 1968b; Gibson *et al.* 1973; Gibson 1976). For example, *Pp*F1 can oxidize and detoxify trichloroethylene (TCE) and transform indole to indigo in the blue dye production process, but both TCE and indole are not able to support *Pp*F1 growth as a carbon source (Wackett & Gibson 1988; Wackett & Householder 1989; Zylstra *et al.* 1989).

1.4 Toluene dioxygenase mediated aromatic hydrocarbons biodegradation

The toluene dioxygenase (TDO) pathway initiates the oxidation of toluene by TodABC that inserts both oxygen molecules into the benzene ring to form toluene-*cis*-2,3-dihyrdodiol (Figure 2). Toluene dioxygenase is a multicomponent enzyme that contains a reductase (TodA), a small iron-sulfur protein (ISP) ferredoxin (TodB) and a large ISP that consists of two subunits ($TodC_1$, $TodC_2$). TodA accepts electron from NADH and passes the electron to TodB. TodB reduces the large ISP that is the terminal oxygenase that catalyzes the oxidation of the toluene. The second enzyme in TDO pathway, toluene-*cis*-dihydrodiol dehydrogenase (TodD) performs dehydrogenation to convert the substrate to form 3-methylcatechol. The next step is a *meta*-fission ring cleavage reaction, which is taken place by catechol-2,3-dioxygenase (C23O or TodE). TodE cleaves the ring at position between C1 and C2 to form 2-hydro-6-oxo-2,4-heptadienoate (HOHD). HOHD is further biodegraded by HOHD hydrolase (TodF) that splits the substrates into a 5 carbons ring opened intermediate and an organic acid, which are cis-2-hydroxypenta-2,4-dienoate and acetic acid, respectively. This 5 carbons ring opened intermediate carries on its TDO metabolism pathway with a hydratase (TodG) to form 3-hydroxy-2-oxo-valerate. The final step of TDO pathway is done by an aldolase (TodH) that breaks the

substrate into acetaldehyde and pyruvate (Figure 2). Both acetaldehyde and pyruvate able to enter the tricarboxylic acid (TCA) cycle and serve as a carbon and energy sources to support the growth of the cells (Yeh *et al.* 1977; Subramanian *et al.* 1979; Subramanian *et al.* 1981; Subramanian *et al.* 1985).

Besides toluene dioxygenase pathway, p-cymene pathway that coverts *p*-cymene to *p*-cumate was also studied in *Pp*F1 (Eaton 1997).

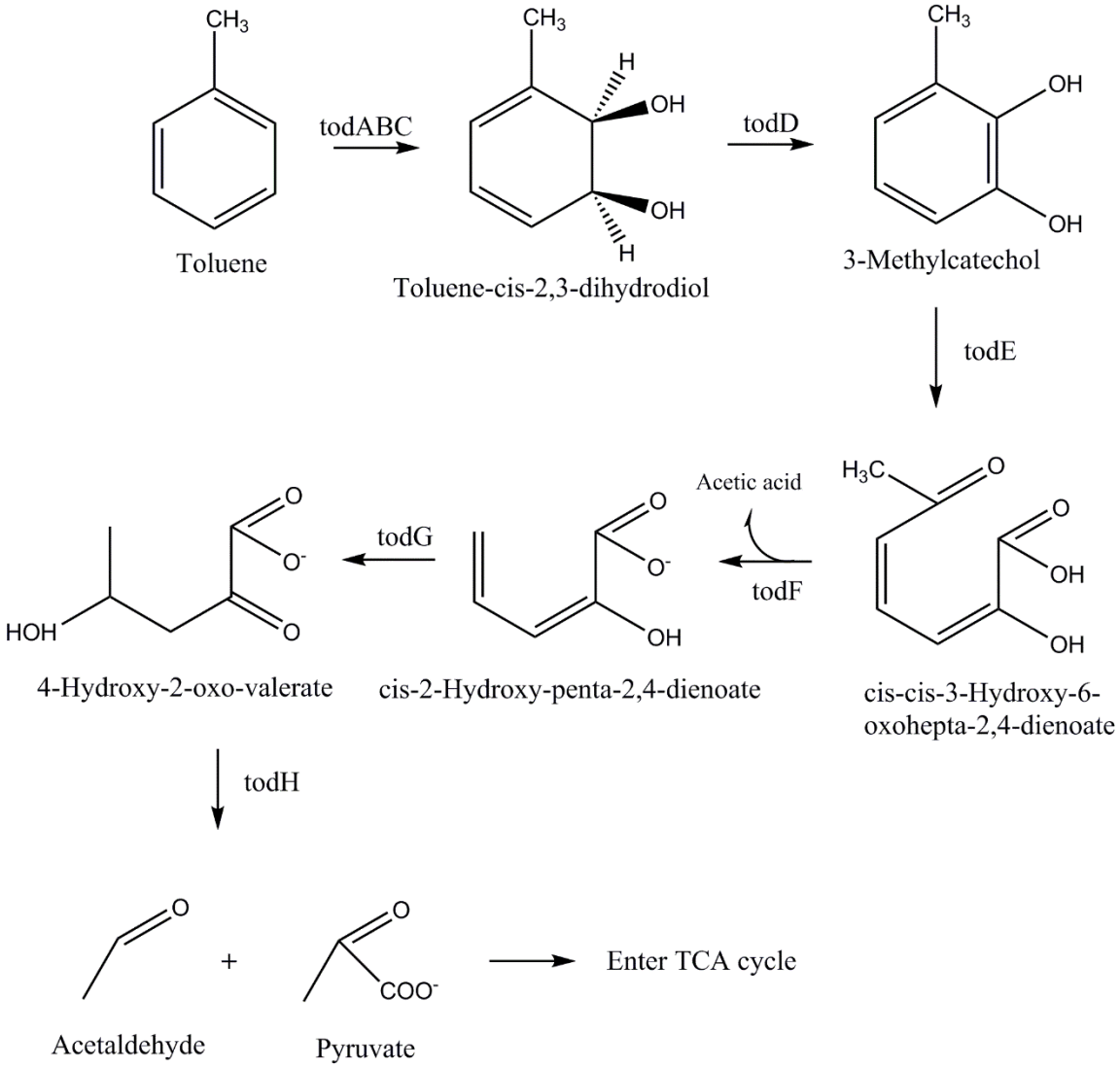

Figure 2. Toluene dioxygenase metabolism pathway. Toluene dioxygenase mediated aromatics degradation pathways in *P. putid*a F1. R group refers to varies derivatives that are listed in the table 2. The organic acids refer to the products that are listed in the table 3. todABC (toluene dioxygenase), todD (toluene cis-dihydrodiol dehydrogenase), todE (catechol-2,3-dioxygenase), todF (meta-fission intermediate hydrolase).

1.5 Toluene dioxygenase substrates

Toluene dioxygenase can oxidize more than 150 aromatic compounds. However, only few of them can be metabolized throughout the whole TDO pathway. The inability to grow on numbers of substrates is in many cases due to the accumulation of catechols that cannot be metabolized further (George *et al.* 2011). In some cases, catechols have been shown to chelate iron out of the catechol-2,3-dioxygenase and inactivate the enzyme. Then, catechol oxygenase activity goes to zero, catechol accumulation occurs, and the cells have not obtained any usable carbon and so they do not grow (George & Hay 2012).

1.6 Genome and genes regulation of TDO pathway in *Pp*F1

The genetics and the operon cluster about the toluene metabolism from *Pp*F1 has been well studied as well (Zylstra *et al.* 1988). The toluene metabolism gene cluster (Figure 3) covers from Pput_2884 to Pput_2872, and 3275718th base pair to 3261582th base pair in terms of DNA sequence. The detail information for each gene and annotation are listed in table 1.

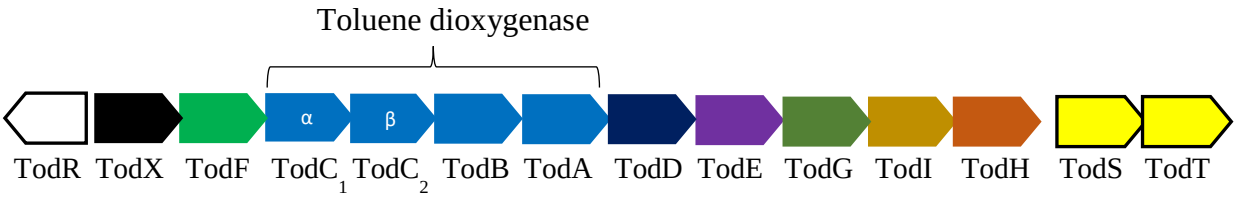

Figure 3. Genetic cluster of toluene metabolism in *Pseudomonas putida* F1

Table 1. Toluene dioxygenase pathway gene cluster

Gene	Protein name	Subcellular localization
todR	LysR family transcriptional regulator	Cytoplasmic
todF	2-hydroxy-6-oxohepta-2,4-dienoate hydroxylase	Unknown
$todC_1$	Toluene dioxygenase subunit alpha	Cytoplasmic
$todC_2$	Toluene dioxygenase subunit beta	Cytoplasmic
todB	Toluene dioxygenase ferredoxin component	Cytoplasmic
todA	Toluene dioxygenase ferredoxin reductase component	Cytoplasmic
todD	Toluene-cis-dihydrodiol dehydrogenase	Cytoplasmic
todE	Catechol-2,3-dioxygenase	Unknown
todG	2-keto-4-pentenoate hydratase	Cytoplasmic
todI	Acetaldehyde dehydrogenase	Cytoplasmic
todH	4-hydroxy 2-oxovalerate aldolase	Cytoplasmic
todS	PAS/PAC sensor hybrid histidine kinase	Cytoplasmic Membrane
todT	Response regulator receiver protein	Cytoplasmic

Besides toluene dioxygenase pathway, there are several aromatic catabolism pathways that have been identified on the chromosome as well (Figure 4). To date, as we have known, it includes *p*-cumate metabolism pathway (Eaton 1997), benzoate metabolism pathway (Mandalakis *et al.* 2013) and phenylacetic acid pathway (Luu *et al.* 2013).

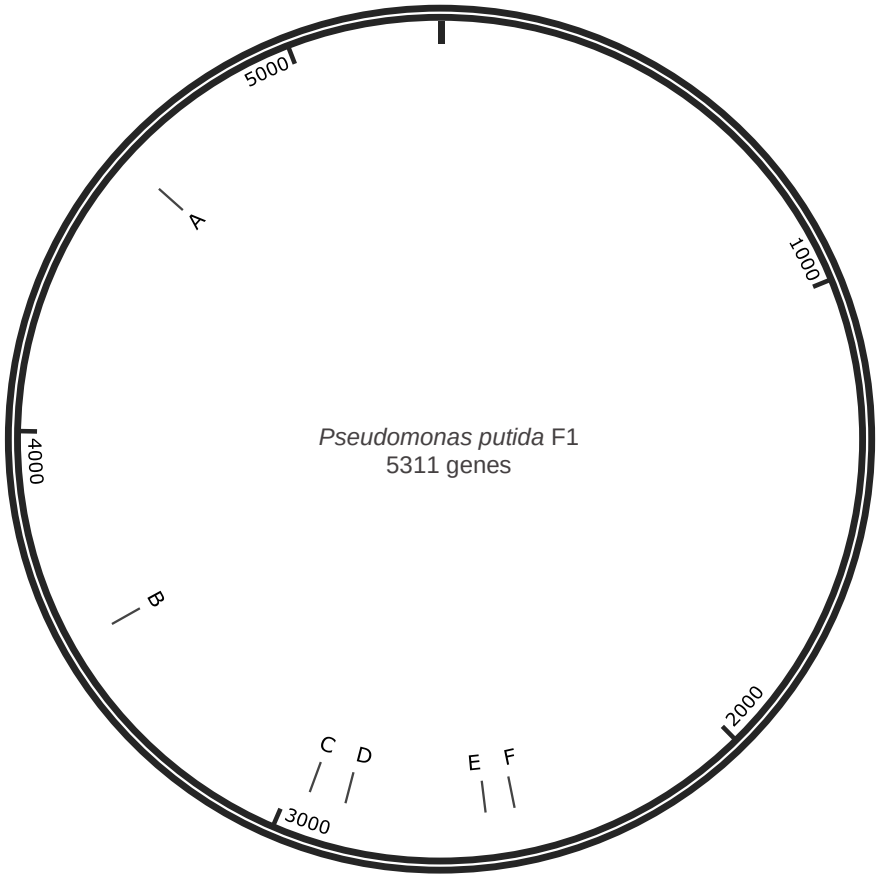

Figure 4. Aromatic compound catabolism island and some pathway related to this study in *Pseudomonas putida* F1. A: Lactic acid metabolism (Pput_4600-4603); B: Formate metabolism (Pput_3551-3554); C: *p*-cumate (Pput_2892-2899); D: Toluene metabolism (Pput_2871-2884); E: Benzoate metabolism (Pput_2548-2554); F: Phenylacetic acid metabolism (Pput_2473-2489).

1.7 Rational for research

Pseudomonas species are capable to survive in diverse nutritional conditions and renowned for their bioremediation capability and potential. To enhance the understanding of the metabolic versatility of *Pp*F1, we studied *Pp*F1 that adapted propylbenzene, a substrate that the wild-type organism normally does not grow on. However, it had been reported that exposing *Pp*F1 to propylbenzene would, over time, allow it to grow. This propylbenzene "adapted" strain had been reported to simultaneously acquire the ability to grow on some other substrates (Choi *et al.* 2003). With this adapted strain, we further explored the capability of *Pp*F1 to assimilate other categories of aromatic hydrocarbons at the C1 position such as alkenylbenzenes, multi-rings, esters, amides, alcohols, amines, and chloroalkylbenzenes.

The present study demonstrated multiple new growth substrates of *P. putida* F1 and showed how growth range might be enlarged in laboratory, and perhaps natural environments. This study showed new growth substrates that have not been tested in the past, and provide knowledge about the multiple ring cleavage strategies that indigenously exist in the propylbenzene-adapted *P. putida* F1. Surprisingly, we not only identified new growth substrates, such as 1-phenylethanol and allylbenzene, but also

their downstream products such as lactic acid and 3-butenoic acid, can also serve to support the cells growth. It was shown that a putative lactic acid operon could be induced when the 1-phenylethanol is supplied as the carbon source. Moreover, 2-phenylacetamide grown cells use malonamic acid or malonamide as the sole nitrogen source for growth, presumably they are intermediates in the metabolism.

In the present study, more than 10 new growth substrates using the toluene dioxygenase mediated pathway were identified. Previously, only seven (benzene, toluene, toluene, ethylbenzene, propylbenzene, butylbenzene, and biphenyl) had been known. This study showed that aromatic hydrocarbon degrading bacteria can have a greater capability for biodegradation and growth than often displayed in typical laboratory experiments (Figure 5).

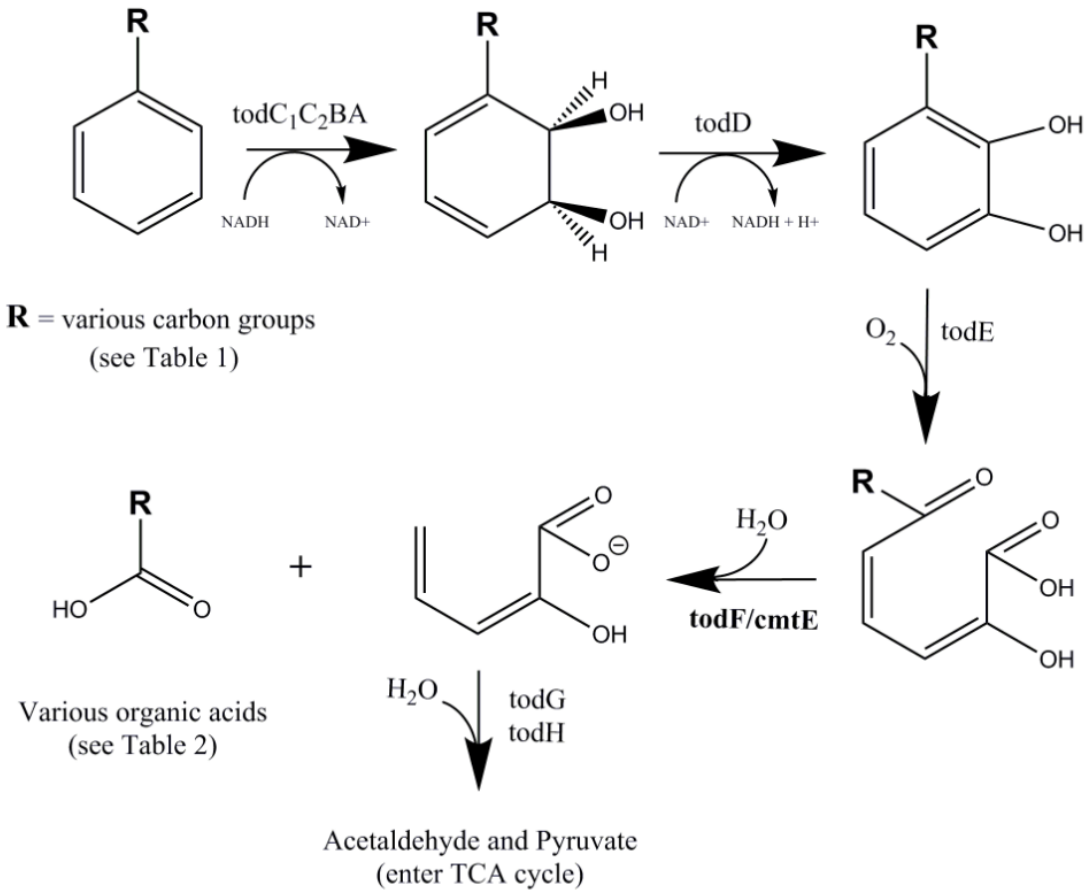

Figure 5. The schematic diagram of the rational of this work.

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CHAPTER 2

PSEUDOMONAS PUTIDA F1 ADAPTS TO METABOLIZE AND GROW ON AROMATIC HYDROCARBONS

2.1 Introduction

*Pp*F1 has been well studied for its ability to assimilate and grow on benzene, benzoate, toluene, ethylbenzene, *p*-cymene and phenylacetic acid. Moreover, it was observed that the strain could readily adapt, presumably through mutation, to grow on biphenyl, *n*-propylbenzene, isopropylbenzene and *n*-butylbenzene (Choi *et al.* 2003). In this study, we greatly expanded the known set of aromatic growth substrates for *Pp*F1. This was done by obtaining a strain of *Pp*F1 that would grow readily on *n*-propylbenzene and then transferring that derived strain into various aromatic compounds.

2.2 Materials and methods

2.2.1 Compounds tested (sources and purities)

Most of the chemicals were purchased from Sigma-Aldrich and TCI. The following chemicals were purchased from Sigma or Aldrich: ethylbenzene (>99%), *n*-propylbenzene (98%), cyclopropylbenzene (97%), isobutylbenzene (99%), cyclohexylbenzene (98%), allylbenzene (98%), 2-phenyl-2-oxazoline (99%), phenyl formate (>98%), phenyl carbamate (97%), phenylacetate (99%), benzamide (99%), phenylurea (97%), phenylboronic acid (97%), benzylamine (99%), acetophenone (99%), benzyl alcohol (>99%), *p*-cresol (99%), 4-methylanisole (99%) and thioanisole (>9%).

The following chemicals were purchased from TCI: *tert*-butylbenzene (>98%), 4-phenyl-1-butene (98%), 1-phenyl-3-pyrazolidone (>98%), 2-chloroethylbenzene (>99%), 2-phenylacetamide (>98%), diphenyl sulfide (>98%) and diphenyl ether (>99%).

With the following exceptions: toluene (Fisher Scientific, 99.9%), tris base (Fisher Scientific, >99%), *n*-butylbenzene (Alfa Aesar, 99%), n-pentylbenzene (Alfa Aesar, 96%), *n*-hexylbenzene (Fluka, analytical grade), diphenylmethane (Alfa Aesar, >99%), 1-chloroethylbenzene (Kodak, >98% by NMR), phenylacetic acid (Eastman) and cinnamyl alcohol (Acros, 98%).

2.2.2 Growth conditions and media

*Pp*F1 was grown with minimal salt base medium (MSB) (20 mM NH_4Cl, 25 mM Na_2HPO_4, 20 mM KH_2PO_4) at pH 6.8 with vapor addition of toluene as a sole carbon source. Each liter of MSB was supplied with 20 ml of Hutner's mineral and vitamin solution (Appendix I). To generate the substrate expanded (adapted) strain, the sole carbon source was switched from toluene to propylbenzene when the cell density reached early log phase (OD_{600} = 0.5 ~ 0.7). All cultures were grown at 30°C with 220 rpm agitation. Aromatic carbon sources were provided either as vapor or direct addition (Table 2 - 8).

2.2.3 Cell density measurement

Cell mass was determined using Beckman (Chaska, MN) DU-6400 spectrophotometer with wavelength 600 nm. The measurement will be used to determine the growth rates, cell density and timing for switching carbon sources. During the enrichment culture, the growth was recorded with the time interval between 8 and 12 hours. All measurements were done by using 1-cm diameter polystyrene disposable cuvette (Sarstedt, Germany).

2.2.4 New growth substrate identification

In addition to few known growth substrates, we tested dozens of aromatic compounds individually that were supplied as a sole carbon source to check if any of them can support the cells growth over time. In this study, several categories of aromatic hydrocarbons were tested, which included alkylbenzenes, alkenylbenzenes, hydroxy-alkylbenzenes, multi-rings compounds, esters, ethers, alcohols, halogenated alkyl chain, ketone, thioesters, amines, amides and urea aromatics. They were added to the medium either in vapor or direct addition method as previously described. For those added directly to the medium, they were supplied as a solid or crystallized powder, and the final concentration was 0.2% (w/v).

Table 2. Alkylbenzenes tested for supporting growth of *Pp*F1

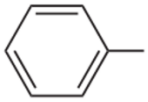
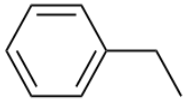
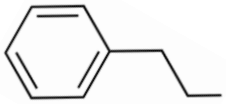
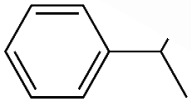
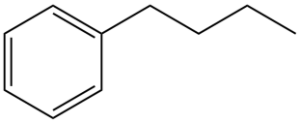
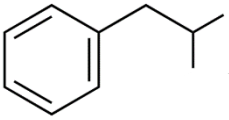
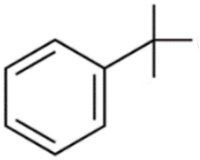
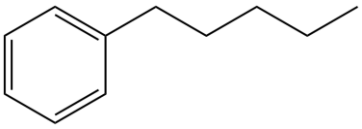
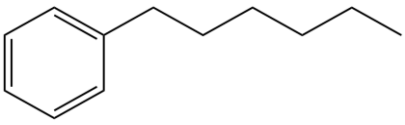
Aromatic compound names	Chemical structures
Toluene	
Ethylbenzene	
n-Propylbenzene	
Isopropylbenzene	
n-Butylbenzene	
Isobutylbenzene	
Tert-butylbenzene	
n-Pentylbenzene	
n-Hexylbenzene	

Table 3. Alkenylbenzenes tested for supporting growth of *Pp*F1

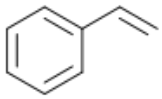
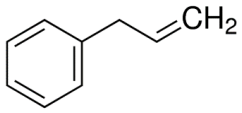
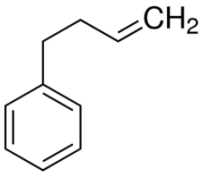
Aromatic compound names	Chemical structures
Styrene	
Allylbenzene	
4-Phenyl-1-butene	

Table 4. Multi-ring aromatics tested for supporting growth of *Pp*F1

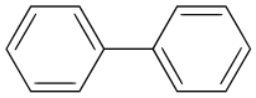
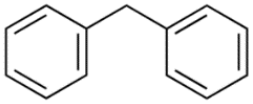
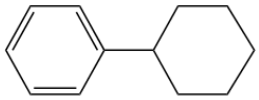
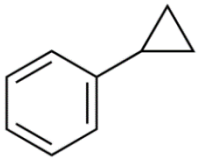
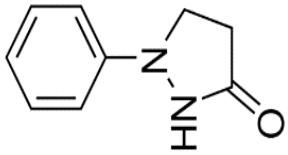
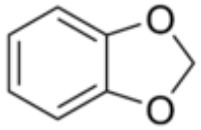
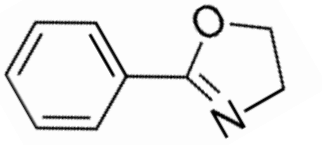
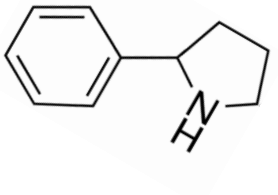
Aromatic compound names	Chemical structures
Biphenyl	
Diphenylmethane	
Cyclohexylbenzene	
Cyclopropylbenzene	
1-Phenyl-3-pyrazolidone	
1,3-Benzodioxole	
2-Phenyl-2-oxazoline	
2-phenylpyrrolidine	

Table 5. Halo- and hydroxy-alkylbenzenes tested for supporting growth of *Pp*F1

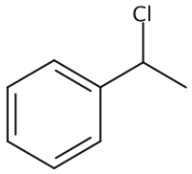
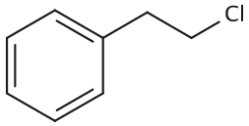
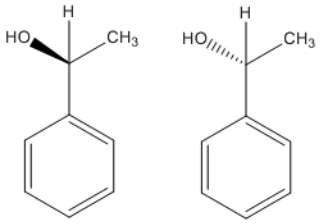
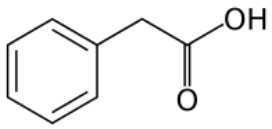
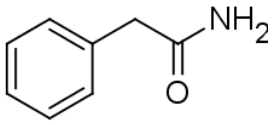
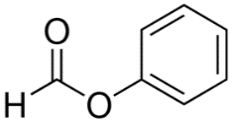
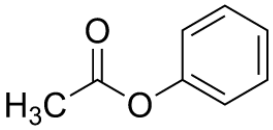
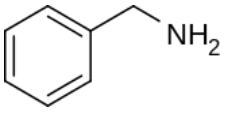
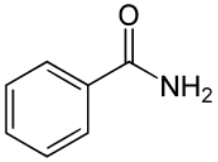
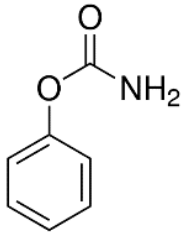
Aromatic compound names	Chemical structures
1-Chloroethylbenzene	
2-Chloroethylbenzene	
1-Phenylethanol (+/-)	

Table 6. Aromatic acids, amides, esters, amines, ureas tested for supporting growth of *Pp*F1

Aromatic compound names	Chemical structures
Phenylacetic acid	
Phenylacetamide	
Phenyl formate	
Phenyl acetate	
Benzylamine	
Benzamide	
Phenyl carbamate	

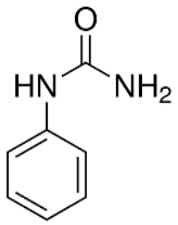
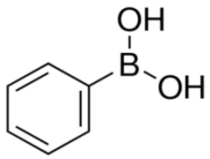
Phenylurea	
Phenylboronic acid	

Table 7. Aromatic ketones, alcohols tested for supporting growth of *Pp*F1

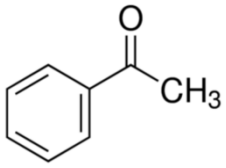
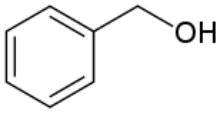
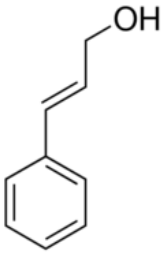
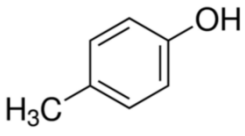
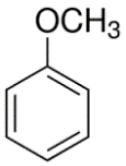
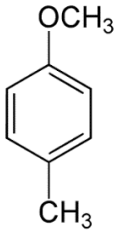
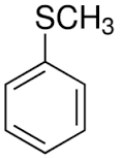
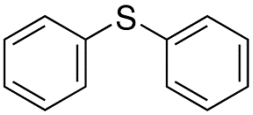
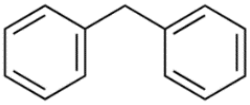
Aromatic compound names	Chemical structures
Acetophenone	
Benzyl alcohol	
Cinnamyl alcohol	
p-Cresol	

Table 8. Aromatic ethers, thioethers tested for supporting growth of *Pp*F1

Aromatic compound names	Chemical structures
Anisole	
4-Methylanisole	
Thioanisole	
Diphenyl sulfide	
Diphenyl ether	

2.3 Results and discussion

2.3.1 Propylbenzene-adapted *Pp*F1

The growth rate of *Pp*F1 was slow at the beginning of the switch of the sole carbon source from toluene to *n*-propylbenzene. However, after several enrichment transfers with supplying *n*-propylbenzene as the sole carbon source, the growth rate became faster and acceptable for further manipulations. The adapted strain is called propylbenzene-adapted *Pp*F1. The first generation of propylbenzene-adapted strain was frozen at -80°C with 8% DMSO supplied.

The growth of propylbenzene-adapted *Pp*F1 was not consistent when inoculants were taken from a -80°C frozen vial of an adapted strain. The growth was typically slow and the media showed a yellowish color in 24-hour cultures. The cells were observed not to grow if the color of the culture turned brown or darker.

To maintain the successfully grown propylbenzene-adapted *Pp*F1 culture, enrichment culture technique was applied. One ml of a culture grown to an OD_{600} of 3.0 absorbance units was transferred to 50 ml fresh MSB with supplying propylbenzene as a sole carbon source and incubating in the same condition as previously.

2.3.2 Expansion of aromatic hydrocarbon substrates

Besides benzene, toluene, ethylbenzene and p-cymene, the propylbenzene-adapted *Pp*F1 can also assimilate other alkylbenzene such as isopropylbenzene, *n*-butylbenzene, *tert*-butylbenzene, *n*-pentylbenzene and *n*-hexylbenzene. In the group of alkenylbenzenes, we found that propylbenzene-adapted *Pp*F1 can use allylbenzene and 4-phenyl-1-butene as a sole carbon source. Besides, propylbenzene-adapted *Pp*F1 feeds on non-fused ring aromatics such as biphenyl, cyclohexanebenzene, cyclopropylbenzene and diphenylmethane. In addition, we identified some other categories of aromatic compounds could be used as a sole carbon source for propylbenzene-adapted *Pp*F1 as well. That included 2-chloroethylbenzene, 1-phenylethanol, 2-phenylacetamide, phenyl formate, phenyl acetate and benzylamine. In contrast, *Pp*F1 couldn't grow on some aromatic acids, multi-rings, amides, esters, amines, urea, ketones, alcohol, ethers and thioethers, such as 1-phenyl-3-pyrazolidone, 1,3-benzodioxole, 2-phenyl-2-oxazoline, 2-phenylpyrrolidine, benzamide, phenyl carbamate, phenylurea, phenylboronic acid, *n*-butyl phenyl ether, acetophenone, benzyl alcohol, cinnamyl alcohol, *p*-cresol, anisole, 3-methylanisole, thioanisole, diphenyl sulfide and diphenyl ether. All aromatic hydrocarbons that had

been tested in this study, and the final absorbance of culture growth experiment, are listed in the Table 9.

In addition to the results that have been listed in Table 9, several aromatic hydrocarbons that showed no growth presented a dark color in the growth medium over time. The dark color was presumed to be due to the accumulation of catechols (dihydroxybenzene), that are known to be relatively toxic intermediate products along the TDO pathway (George *et al.* 2011; George & Hay 2012). For this reason, 1 % (w/v) XAD-4 resin (Aldrich, 20-60 mesh) was applied for culture medium to adsorb catechols and reduce their toxicity. This was done with 3-methoxycatechol, 1-phenyl-3-pyrazolidine, γ-phenyl-γ-butyrolactone and acetophenone. Among these aromatic hydrocarbons, only γ-phenyl-γ-butyrolactone grew steadily over time in the presence of XAD-4 resin. The yellowish color in the medium was also significantly reduced, which indicated success in removing the catechol by the XAD-4 resin. The XAD-4 resin might have to adsorb only excess catechols, and some catechol must be further metabolized to support growth of the cells.

2-phenylacetadmide had been known for supporting Pseudomonas species growth (Betz & Clarke 1973). In this study, we further investigated the metabolism pathway in propylbenzene-adapted *Pp*F1.

Table 9. Growth of *P. putida F1* derivative on aromatic compounds, showing relative extent of growth as measured by absorbance (OD) at 600 nm after 24 h. Generally, volatile liquids were supplied as a vapor and solids were supplied by direct addition to the medium.

Growth substrate tested	Method of adding substrates	ΔOD_{600} at 24hr
	Alkylbenzenes	
Toluene (positive control)	Vapor	1.8
Ethylbenzene	Vapor	1.4
n-Propylbenzene	Vapor	1.1
Isopropylbenzene	Vapor	1.2
n-Butylbenzene	Vapor	1.2
Isobutylbenzene	Vapor	1.4
Tert-butylbenzene	Vapor	0.6
n-Pentylbenzene	Vapor	0.7
n-Hexylbenzene	Vapor	1.4
	Alkenylbenzenes	
Allylbenzene	Vapor	1.4
4-Phenyl-1-butene	Vapor	0.5
4-Phenyl-1-butene	Direct addition	0.9
	Multi-ring aromatics	
Biphenyl	Direct addition	1.4
Diphenylmethane	Direct addition	1.3
Cyclohexylbenzene	Direct addition	0.5
Cyclopropylbenzene	Direct addition	0.3
1-Phenyl-3-pyrazolidone	Direct addition	<0.1
1,3-Benzodioxole	Direct addition	<0.1
2-Phenyl-2-oxazoline	Direct addition	<0.1
2-phenylpyrrolidine	Direct addition	<0.1
	Halo- and hydroxy-alkylbenzenes	
1-Chloroethylbenzene	Vapor	1.5
2-Chloroethylbenzene	Vapor	0.5
1-Phenylethanol (+/-)	Vapor	1.8

Aromatic acids, amides, esters, amines, urea		
Phenylacetic acid	Direct addition	2.4
Phenylacetamide	Direct addition	1.9
Phenyl formate	Vapor	1.1
Phenyl acetate	Vapor	1.1
Benzylamine	Vapor	0.7
Benzamide	Direct addition	<0.1
Phenyl carbamate	Direct addition	<0.1
Phenylurea	Direct addition	<0.1
Phenylboronic acid	Direct addition	<0.1
Aromatic ketones, alcohols		
Acetophenone	Vapor	<0.1
Benzyl alcohol	Vapor	<0.1
Cinnamyl alcohol	Vapor	<0.1
p-Cresol	Vapor	<0.1
Aromatic ethers, thioethers		
Anisole	Vapor	<0.1
4-Methylanisole	Vapor	<0.1
Thioanisole	Vapor	<0.1
Diphenyl sulfide	Vapor	<0.1
Diphenyl ether	Vapor	<0.1

2.3.3 Chemical features of growth substrates and non-growth substrates

Among those aromatic growth substrates and aromatic non-growth substrates, there are some features we can differentiate them by comparing to each other. It is better to visualize and compare them side-by-side with their chemical structures (Figure 6.)

In the list of growth substrates, 4-phenyl-1-butene, benzylamine, allylbenzene, ethylbenzene and cyclohexylbenzene *et cetera*, can support the propylbenzene adapted *Pp*F1 growth. In contrast, cinnamyl alcohol, benzamide, styrene and 2-phenyl-2-oxazoline *et cetera*, could not support propylbenzene adapted *Pp*F1 in this study.

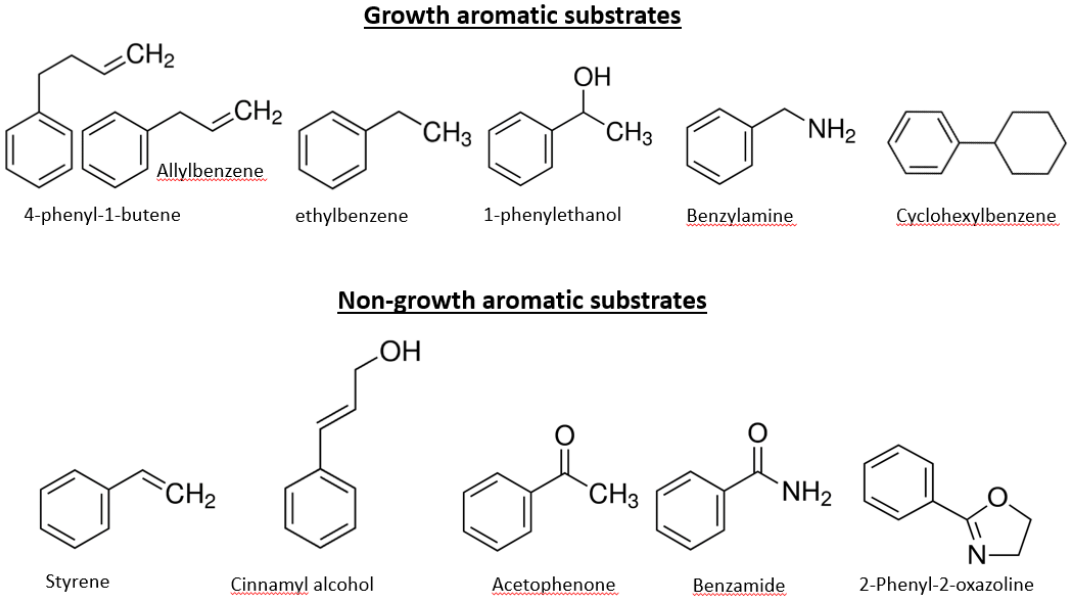


Figure 6. Chemical properties between some of the growth and non-growth substrates.

Most of the aromatic compounds having a saturated benzylic carbon adjacent to the benzene ring are growth substrates (Figure 6, first row). However, aromatic compounds that contain a benzylic carbon double bonded to a carbon, oxygen or nitrogen are not growth substrate (Figure 6, second row).

Styrene is an example of an aromatic hydrocarbon known to be a substrate of toluene dioxygenase (Boyd *et al.* 2005), but it cannot serve as a growth substrate for wild-type *Pp*F1 due to the inability of catechol-2,3-dioxygenase in the TDO pathway to catalyze the ring-opening reaction (George *et al.* 2011; George & Hay 2012). On the other hand, allylbenzene and 4-phenyl-1-butene are just one and two carbon difference from the styrene on the alkenyl chain subtitution. They both can support the propylbenzene-adapted *Pp*F1 growth as a sole carbon source.

According to the features between growth substrates and non-growth substrates in this study. We hypothesize either the double bond adjunct to the benzylic carbon or the carbonyl carbon could play a critical rule in the failure of being metabolized through TDO pathway. And it is very possible that the metabolic step limiting growth is that catalyzed by catechol-2,3-dioxygenase.

2.4 References

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CHAPTER 3

ORGANIC ACIDS PRODUCED BY OXIDATION OF AROMATIC COMPOUNDS TESTED AS CARBON SOURCE.

3.1 Introduction

*Pp*F1 can degrade aromatic hydrocarbons and produce organic acids during metabolism by the TDO pathway (Kasahara *et al.* 2012). This organic acid intermediate will be split from the *meta*-fission intermediate product by TodF – the *meta*-fission intermediate hydrolase (Menn *et al.* 1991; Choi *et al.* 2003). By supplying the organic acid as a sole carbon source, we can learn whether it can be a carbon source or toxic to the cells. By doing so, we learn if the aromatic hydrocarbons are getting completely metabolized via the TDO pathway. For example, if we can detect lactic acid in the culture media by supplying 1-phenylethanol as a sole carbon source, it suggests not only the lactic acid is the product of TodF hydrolysis reaction of *meta*-fission intermediate, but also strongly indicates that 1-phenylethanol is metabolized via TDO pathway. However, besides lactic acid, many of the hypothesized TodF produced organic acids don't have a simple method a detect them. For this reason, lactic acid was one of the representative organic acid that we studied in some detail.

3.2 Materials and methods

3.2.1 Compounds tested (sources and purities)

Most of the chemicals were purchased from Sigma-Aldrich and TCI. The following chemicals were purchased from Sigma or Aldrich: *n*-pentanoic acid (~99%), 3-methylbutanoic acid (99%), 2,2-dimethylpropionic acid (99%), 4-pentenoic acid (97%), benzoic acid (>99%), cyclohexanoic acid (98%), R-(+)-2-chloropropanoic acid (99%), and S-(-)-2-chloropropanoic acid (97%), D-(-)-lactic acid (95-100%), L-(+)-lactic acid (>98%).

The following chemicals were purchased from TCI: isobutanoic acid (>99%), n-hexanoic acid (>98%), n-heptanoic acid (>98%), and 3-chloropropanoic acid (>98%).

With the following were purchased from other companies: D-lactic acid (Oakwood Chemicals, 90%), propanoic acid (Eastman), n-butanoic acid (Fluka, >99.5%), 3-butenoic acid (90%), cyclopropanoic acid (98%) and phenylacetic acid (Eastman).

3.2.2 Growth condition and media

*Pp*F1 was grown with minimal salt base medium (MSB) (20 mM NH_4Cl, 25 mM Na_2HPO_4, 20 mM KH_2PO_4) and 20 ml of Hutner's mineral and vitamin solution for each 1 liter of MSB. 0.2% organic acid (w/v) was added by direct addition and serves as a sole carbon source. The pH of the medium was adjusted by NaOH to 6.8 before cells are inoculated. The initial optical density of inoculated was 0.1. All the growth measurements were performed by visible light spectrophotometer at optical density 600 nm. The final OD indicates the difference between initial and final absorbance.

3.2.3 New growth substrate identification

In addition to common organic acids such as succinate, that can be used as growth substrates, we tested dozens of organic acids that corresponding to their aromatic hydrocarbons precursors that would be generated during aromatic metabolism (Table 10-13).

Table 10. Alkanoic acids tested for supporting growth of *Pp*F1

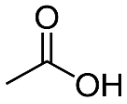
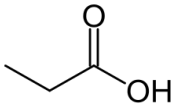
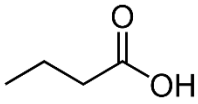
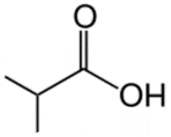
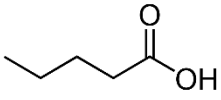
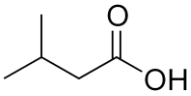
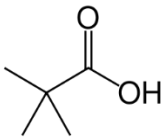
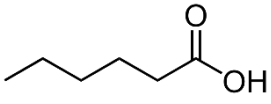
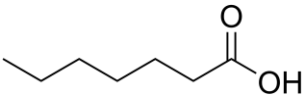
Organic acid names	Chemical structures
Acetic acid	
Propanoic acid	
n-Butanoic acid	
Isobutanoic acid	
n-Pentanoic acid	
3-Methylbutanoic acid	
2,2-Dimethylpropionic acid	
n-Hexanoic acid	
n-Heptanoic acid	

Table 11. Alkenoic acids tested for supporting growth of *Pp*F1

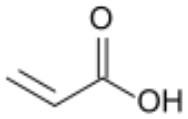
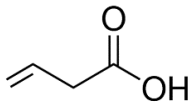
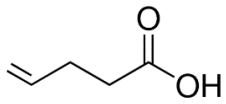
Organic acid names	Chemical structures
Acrylic acid	
3-Butenoic acid	
4-Pentenoic acid	

Table 12. Haloalkanoic acids tested for supporting growth of *Pp*F1

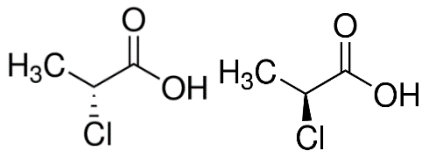
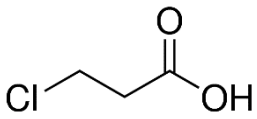
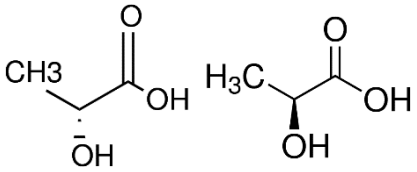
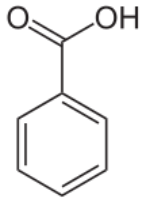
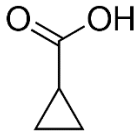
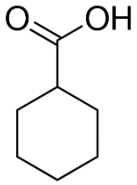
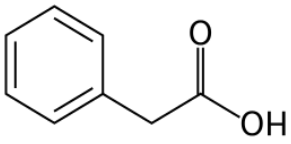
Organic acid names	Chemical structures
(R/S)-2-Chloropropanoic acid	
3-Chloropropanoic acid	
(D/L)-Lactic acid	

Table 13. Ring carboxylic acids tested for supporting growth of *Pp*F1

Organic acid names	Chemical structures
Benzoic acid	
Cyclopropanoic acid	
Cyclohexanoic acid	
Phenylacetic acid	

3.3 Results and discussion

In addition to those we have known in the past, we identified other novel organic acids could serve as a sole carbon source. Those include *n*-butanoic acid, isobutanoic acid, *n*-pentanoic acid, 2-methylbutanoic acid, *n*-hexanoic acid, *n*-heptanoic acid, 3-butenoic acid, benzoic acid, phenylacetic acid, L-lactic acid and D-lactic acid. The details are summarized in Table 14. Decanoic acid, nonanoic acid and octanoic acid were also tested and shown support propylbenzene-adapted *Pp*F1 growth. They are not listed in the table as a part of result because their aromatic precursors were not available to test for growth.

Some of the organic acids in the list couldn't support growth, even though their aromatic precursors do. This includes 2,2-dimethylpropionic acid, 4-pentenoic acid, arylic acid, R-(+)-2-chloropropionic acid, S-(-)-2-chloropropionic acid, 3-chloropropionic acid and glycine. It suggested that the growth is majorly relies on the pyruvate and acetaldehyde produced from 2-hydroxypenta-2,4-dienoate – the 5 carbons intermediate product from TodF. The reason is still unclear why *Pp*F1 can assimilate 3-butenoic acid but not 4-pentenoic acid. All the growth studies were replicated to confirm the results.

Table 14. Growth of *P. putida* F1 on organic acids that derived from aromatic substrates, showing relative extent of growth.

Growth substrate tested	Aromatic precursor	ΔOD_{600}
	Alkanoic acids	
Acetic acid	Toluene	3.5
Propanoic acid	Ethylbenzene	1.4
n-Butanoic acid	*n*-Propylbenzene	1.3
Isobutanoic acid	Isopropylbenzene	1.0
n-Pentanoic acid	*n*-Butylbenzene	1.2
3-Methylbutanoic acid	Isobutylbenzene	2.6
2,2-Dimethylpropionic acid	*Tert*-butylbenzene	<0.1
n-Hexanoic acid	*n*-Pentylbenzene	1.0
n-Heptanoic acid	*n*-Hexylbenzene	4.5
	Alkenoic acids	
Acrylic acid	Styrene	<0.1
3-Butenoic acid	Allylbenzene	1.2
4-Pentenoic acid	4-Phenyl-1-butene	<0.1
	Ring carboxylic acids	
Benzoic acid	Biphenyl	0.6
Cyclopropanoic acid	Cyclopropylbenzene	<0.1
Cyclohexanoic acid	Cyclohexylbenzene	<0.1
Phenylacetic acid	Diphenylmethane	2.4
	Haloalkanoic acids	
R-(+)-2-Chloropropanoic acid	1-Chloroethylbenzene	<0.1
S-(–)-2-Chloropropanoic acid	1-Chloroethylbenzene	<0.1
3-Chloropropanoic acid	2-Chloroethylbenzene	<0.1
D/L-Lactic acid[1]	-	<0.1
L-Lactic acid[2]	1-Phenylethanol	2.0
D-Lactic acid[2]	1-Phenylethanol	0.2
	Amino acid	
Glycine	Benzylamine	<0.1

[1] Direct addition to the cells before getting exposed to 1-phenylethanol. [2] After exposed to 1-phenylethanol.

3.4 References

Choi E.N., Cho M.C., Kim Y., Kim C.K. & Lee K. (2003) Expansion of growth substrate range in *Pseudomonas putida* F1 by mutations in both cymR and todS, which recruit a ring-fission hydrolase CmtE and induce the tod catabolic operon, respectively. *Microbiology* **149**, 795-805.

Kasahara Y., Morimoto H., Kuwano M. & Kadoya R. (2012) Genome-wide analytical approaches using semi-quantitative expression proteomics for aromatic hydrocarbon metabolism in *Pseudomonas putida* F1. *J Microbiol Methods* **91**, 434-42.

Menn F.M., Zylstra G.J. & Gibson D.T. (1991) Location and sequence of the todF gene encoding 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase in *Pseudomonas putida* F1. *Gene* **104**, 91-4.

CHAPTER 4

DEMONSTRATING META-FISSION INTERMEDIATE USING *E. COLI* pDTG351 EXPRESSING *TODC$_1$C$_2$BADE* GENES

4.1 Introduction

To verify aromatic substrates tested were metabolized via the TDO pathway, we used a recombinant *E. coli* strain pDTG351 that carries and expresses *todC$_1$C$_2$BADE* genes encoding the first 3 enzymes in the TDO pathway for this part of experiment (Zylstra *et al.* 1988; Zylstra & Gibson 1989). Through *todC$_1$C$_2$BADE*, the aromatic hydrocarbon substrates are oxidized by toluene dioxygenase (TodC$_1$C$_2$BA), *cis*-dihydrodiol dehydrogenase (TodD) and catechol-2,3-dioxygenase (TodE) to generates a *meta*-fission ring-opened product. This "ring-opened" intermediate has a maximum absorbance at 388 nm and shows a yellow color in the liquid medium, at basic pH. This makes it very convenient to screen for and demonstrate aromatic substrates are indeed being metabolized by the toluene dioxygenase pathway in *Pp*F1.

4.2 Materials and methods

4.2.1 Growth condition and *meta*-fission reaction

E. coli pDTG351 was streaked out from -80°C freezer culture onto a Luria Broth (LB) agar plate with 50 µg/ml kanamycin supplied as a resistance selection and grown overnight at 37°C. Single colony was picked and inoculated into 3 ml LB with 50 µg/ml kanamycin supplied as a pre-culture for overnight culture. The culture was then scaled-up to 50 ml and grown to OD_{600} at 2.0. Culture media aliquots were redistributed to glass tubes and supplied with different aromatic hydrocarbons that have been tried in the previous section for 48 hours with 250 rpm agitation in 30°C incubation room. The cell pellets were spun down and discarded. Supernatants from each sample was retained for spectrophotometric analysis.

The *meta*-fission end products will be formed only if the aromatic hydrocarbon can be first oxidized by toluene dioxygenase, oxidized by *cis*-dihydrodiol dehydrogenase and oxidized by catechol-2,3-dioxygenase to form the yellow "ring-open" *meta*-fission product. This meta-fission product will be accumulated since there is no enzymes from the *Pp*F1 TDO pathway in the recombinant *E. coli* strain.

4.2.2 Sample preparation

By using 96-wells UV microplate (Thermo Fisher Scientific, Waltham, MA), 200 µl supernatant from each sample was aliquoted into 3 wells. Among these 3 wells, the first well serves as a control for each sample without any change. For the second well, the pH will be adjusted by adding 10 µl of 10 N HCl to equal or lower than pH 4.0. And the pH will be adjusted by adding 20 µl of 10 N NaOH to ensure pH is getting higher than pH 10.0 for the third well sample.

4.2.3 UV-visible microplate spectrophotometry

Spectrum scanning from 250 nm to 550 nm was performed by using ELISA microplate reader SpectraMax plus 384 (Molecular Devices, Sunnyvale, CA). Raw data was generated from microplate reader and plotted into a diagram with intensity over wavelength for visualize the UV-vis spectrum.

4.3 Results and discussion

In this assay, toluene was used as a position control and phenylacetic acid was used as a negative control (Figure 7.). The solid black line indicates the supernatant that has no pH adjustment. In some cases, there is an obvious 388 nm signal before the adjustment to basic pH because the pH of the culture increased from pH 6.8 to pH 8.0 during the incubation with many of the aromatic hydrocarbons. The 388 nm signal then increased when the pH was adjusted even higher using NaOH. The high pH makes sure that all of the ring-opened *meta*-cleavage product, if present, is in the tautomeric form that absorbs at 388 nm.

The results of this assay are organized in Table 15. Almost all the results are matching to the previous assay in section 2.3.2. Samples that showed positive and obvious 388 nm spectrum in basic pH are toluene, allylbenzene, 4-phenyl-1-butene, *tert*-butylbenzene, 1-phenylethanol, phenyl formate, phenyl acetate, 2-chloroethylbenzene, benzylamine, *n*-butyl phenyl ether, styrene, acetophenone, cyclohexylbenzene, cyclopropylbenzene, diphenylether, biphenyl, gamma-phenyl-gamma-butyrolactone and diphenyl ether. Others, such as *n*-butyl phenyl ether, diphenyl ether, acetophenone, and styrene presented strong 388 nm signal in basic pH (supplementary data),

but didn’t support growth of the propylbenzene-adapted *Pp*F1 when supplied as a sole carbon source.

In contrast, 2-phenylacetamide can support *Pp*F1 growth as a sole carbon source and nitrogen source (Betz & Clarke 1973), but it does not show signal at 388 nm in this assay. For this reason, 2-phenylacetamide may have a different pathway by which it is metabolized, instead of the TDO pathway. For example, phenylacetic acid has been known to be degraded via an epoxide oxidation pathway that totally different from TDO pathway.

The detail spectrum of each sample is provided in the supplementary data (Chapter 9).

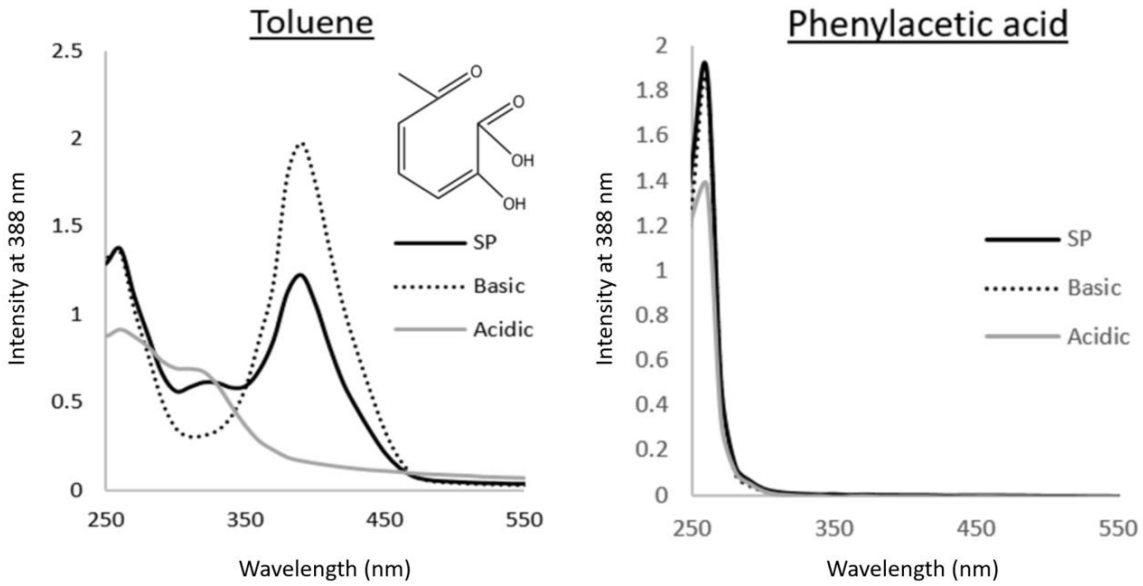
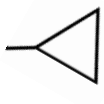
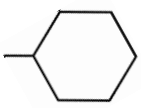
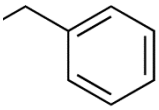
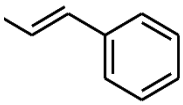
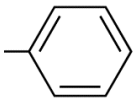
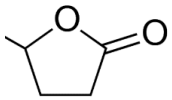
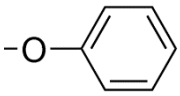
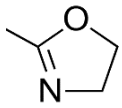
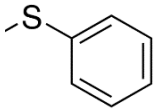
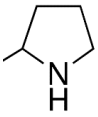


Figure 7. The symbolic patterns spectrum of *meta*-fission product formation between toluene (positive control, left) and phenylacetic acid (negative control, right).

Table 15. *Meta*-fission product assay by *E.coli* pDTG351 (*TodC$_1$C$_2$BADE*) and the growth of propylbenzene-adapted *Pp*F1.

R group	Compounds	λ_{max} = 388 nm	Growth*
–CH_3	Toluene[1]	+	+
–CH_2CH_3	Ethylbenzene	+	+
–$CH_2CH_2CH_3$	Propylbenzene	+	+
–$CH_2CH{=}CH_2$	Allylbenzene	+	+
–$CH_2CH_2CH{=}CH_2$	4-phenyl-1-butene	+	+
–$C(CH_3)_3$	Tert-butylbenzene	+	+
–$CH(OH)CH_3$	1-Phenylethanol[2]	+	+
–OC(O)H	Phenyl formate	+	+
–$OC(O)CH_3$	Phenyl acetate	+	+
–CH_2CH_2Cl	2-chloroethylbenzene	+	+
–CH_2NH_2	Benzylamine	+	+
–OCH_3	Anisole	+	–
–$O(O)NH_2$	phenyl carbamate	+	–
–$OCH_2CH_2CH_2CH_3$	n-Butyl phenyl ether	+	–
–$CH{=}CH_2$	Styrene	+	–
–$C(O)CH_3$	Acetophenone	+	–
–$NHC(O)NH_2$	Phenylurea	–	–
–$C(O)NH_2$	Benzamide	–	–
–$CHCHCH_2OH$	Cinnamyl alcohol	–	–
–OH, –CH_3 (para)	*p*-cresol	–	–
–$B(OH)_2$	Phenylboronic acid	–	–
–C(O)H	Benzylaldehyde	–	–
–$OC(O)NH_2$	Phenyl carbamate	–	–
–OCH_3, –CH_3 (para)	4-methylanisole	–	–
–SCH3	Thioanisole	–	–
–$CH_2C(O)OH$	Phenylacetic acid[3]	–	+
–$CH_2C(O)NH_2$	2-phenylacetamide	–	+
	Cyclopropylbenzene	+	+

	Cyclohexylbenzene	+	+
	Diphenylmethane	+	+
	Stilbene	+	–
	Biphenyl	+	+
	γ-phenyl-γ-butyrolactone	+[4]	+
	Diphenyl ether	+	–
	2-phenyl-2-oxozaline	–	–
	Phenylsulfide	–	–
	2-phenylpyrrolidine	–	–

*Indicates supporting growth for propylbenzene-adapted *Pp*F1 strain.
[1]Positive control; [2]provided as vapor only; [3]phenylacetic acid pathway; [4]with XAD-4 resin support.

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CHAPTER 5

CHARACTERIZATION OF METABOLISM OF 1-PHENYLETHANOL AND 2-PHENYLACETAMIDE IN *P. PUTIDA* F1.

5.1 Introduction

5.1.1 Determining the extent of 1-phenylethanol metabolism via the TDO pathway

One of the steps in the TDO pathway is performed by a *meta*-fission intermediate hydrolase, TodF, that releases an organic acid product (Menn *et al.* 1991). The presence of a specific organic acid that matches to the product from *meta*-fission intermediate from TodF offers a strong indication that if the aromatic hydrocarbons are metabolized through the TDO pathway. However, most of the organic acids derived from the aromatic hydrocarbons are not easily detected and assayed. Fortunately, in the study of 1-phenylethanol metabolism in *Pp*F1 as a case study, lactic acid is the organic acid predicted to be released upon metabolism through the TDO pathway and there are available methods to assay the level of lactic acid and the enzyme activity for NAD-dependent lactate dehydrogenase (LDH) (Zhao *et al.* 2013;(Gao *et al.* 2012; Gao *et al.* 2015).

In the previous growth studies, 1-phenylethanol supported growth of the propylbenzene-adapted *Pp*F1 as a sole carbon source. Previously, 1-phenylethanol had been studied with *Pp*F1 that established toluene dioxygenase as the initiating enzyme in the 1-phenylethanol biodegradation pathway (Bui 2003). In Bui's work, *Pp*F39/D and *E. coli* pDTG601a had been applied to perform the results that both enantiomers of 1-phenylethanol can be oxidized and formed the *cis*-dihydrodiol products by toluene dioxygenase. In an even earlier study, 1-phenylethanol had been shown to be a minor product from hydroxylation of ethylbenzene at the benzylic position (Gibson *et al.* 1973). The 1-phenylethanol thus produced could be further oxidized to form *cis*-3-(1-hydroxyethyl)-3.5-cyclohexadiene-1,2-diol.

If the cis-diol intermediate of 1-phenylethanol is further metabolized by subsequent enzymes in the TDO pathway, we would predict that lactic acid would be formed by the enzyme TodF. Lactic acid might be oxidized by *Pp*F1 to form pyruvate, that would enter the citric acid cycle energy source and supply carbon and energy, or it might accumulate and not be further metabolized. To investigate this, I measured the level of the lactic acid in the culture media while 1-phenylethanol was supplied as a sole carbon source. Additionally, I also assayed cell extract for lactate dehydrogenase (LDH) activity.

5.1.2 Determining the metabolic pathway for 2-phenylacetamide

In the previous growth studies, 2-phenylacetamide could support propylbenzene-adapted *Pp*F1 growth as a sole carbon and nitrogen source. Other *Pseudomonas* strains also grow on this substrate (Clarke 1973). *Pp*F1 could liberate nitrogen as ammonia either of two possible ways: (1) amidase hydrolyzes the amine group from the 2-phenylacetamide as the first reaction, and the metabolism of phenylacetate by a pathway other than the TDO pathway. Or (2) via TDO pathway. By the latter mechanism, toluene dioxygenase initially oxidizes 2-phenylacetamide, followed by a series of TDO pathway enzymatic reactions sequentially. If that is the case, malonamic acid would be released by the TodF reaction. Then, the enzyme malonamidase would release ammonia to obtain the nitrogen to support cell growth.

According to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, 2-phenylacetamide is an intermediate product in phenylalanine metabolism in other bacteria (Appendix II). The amine group of the 2-phenylacetamide could potentially be hydrolyzed by any one of the four amidases [EC:3.5.1.4] that have been annotated to be present in the *Pp*F1 genome (The locus entries of these amidases are Pput_2141, Pput_2609, Pput_2727 and Pput_2760). If those metabolize 2-

phenylacetamide, then the phenylacetate pathway would carry on the rest of the metabolism into acetyl-CoA or succinyl-CoA that enter the citric acid cycle as an energy source. Besides, there were other reports suggested that mandelamide hydrolase could perform the amine group hydrolysis on 2-phenylacetamide (McLeish *et al.* 2003; Gopalakrishna *et al.* 2004).

In this section of study, we tried to determine what pathways operate.

5.2 Materials and methods

5.2.1 Chemicals and buffers

The following were purchased from Sigma or Aldrich: phenylmethylsulfonyl fluoride (PMSF), Benzonase® nuclease, L-lactate dehydrogenase (L-LDH, 5 mg/ml, ~550 units/mg), D-lactate dehydrogenase (D-LDH, unknown volume and unit/volume), N-cyclohexyl-3-aminopropanesulfonic acid buffer (CAPS, 150 mM, pH 9.0), β-NAD (6 mM in CAPS, pH 9.0). Bovine albumin serum (BSA) and Bradford protein assay reagent were purchased from Bio-Rad (Hercules, CA). Lysis buffer contains 50 mM Tris, 100 mM NaCl, 1 mM DTT, the pH was adjusted to pH 8.0.

Solvent ethyl acetate (Sigma, 99.9%), MTBE (Sigma, 99.9%), succinic acid (Sigma), malonamide (Acros, 97%) and malonic acid (Acros, 95%). Malonamic acid was chemically synthesized by Jack Richman.

5.2.2 Strains and plasmids

The *Pseudomonas putida* F1 knockout strains and recombinant plasmid of *TodC_1C_2BA* in *E. coli* are listed at Table 16.

Table 16. Bacterial strains and plasmids used in this work.

Strains	Relevant properties	Reference
Escherichia coli		
pDTG601A	Amp^r *todC_1C_2BA*	Gibson *et al*, 1989.
Pseduomonas putida F1		
*Pp*F1	Tol$^+$ prototroph	This study
Propylbenzene adapted PpF1		This study
*Pp*F04	Tol$^-$ *tod*C_1	Finette *et al*, 1984.
*Pp*F106	Tol$^-$ *tod*C_2	Finette *et al*, 1984.
*Pp*F39/D	Tol$^-$ *tod*D	Gibson *et al*, 1973.
RLF1	Δ*paaF*	Luu *et al*, 2013.
RLF2	Δ*paaI*	Luu *et al*, 2013.

5.2.3 Growth condition

For the samples preparation of lactate assay and LDH enzymatic assay, propylbenzene-adapted *Pp*F1 culture was initially grown at the optical density (OD) of 600 nm at 0.1 at a 250-ml conical flask with 50-ml MSB, with propylbenzene supplied as a sole carbon source. The culture medium was incubated at 30°C with 250 rpm agitation for 36 hours to reach 0.6 at

OD_{600}. Then carbon source was switched to 1-phenylethanol and incubated for another 36 hours. The culture medium was centrifuged at 4000 rpm for 20 minutes. Both supernatant and cell pellet from the propylbenzene-adapted *Pp*F1 culture was harvested while OD_{600} reached 2.4. The supernatant was used for lactic acid assay and the cell pellet was suspended and cells lysed for enzymatic assays.

In determine whether propylbenzene-adapted *Pp*F1 could assimilate 2-phenylacetamide as an energy source and support the growth, several gene-knockout strains were used in the growth study, such as *Pp*F04, *Pp*F106, RLF1 and RLF2. *E. coli* strains pDTG601A and TodD knockout strain *Pp*F39/D were applied for resting cell assay. *E. coli* strain pDTG601A was streaked on LB plate with 100 μg/ml of ampicillin. A single colony was picked and inoculated into LB with 100 μg/ml of ampicillin for overnight growing at 37°C with 250 rpm agitation. The culture medium was centrifuged and the cell pellet was collected and weighted. *Pp*F39/D mutant was streaked on LB plate without antibiotic supplied. A single colony was picked and inoculated into LB for overnight culture before scaled up to 50 ml LB culture. The culture medium was centrifuged after 24-hours incubation with the same condition. The cell pellet was collected and weighted for further manipulation.

5.2.4 Resting cell assay and gas chromatography analysis

0.1 grams per ml of cells were suspended in phosphate-buffered saline (PBS) that contains 10 mM PO_4^{3-}, 100 mM NaCl, and 3 mM KCl (pH 7.4). 3 ml of the appropriate cell suspension was incubated with 6 mg of 2-phenylacetamide in a cap-sealed glass vial for several time points, which included 30, 60, 180 minutes and overnight. Each sample was added 1:1 (v/v) ethyl acetate for extraction.

Resting cell assay samples analyzed by gas chromatography/mass spectrometry/flame ionization detection (GC/MS/FID). The ethyl acetate layer was analyzed by GC/MS/FID. Separation was achieved on an HP-1ms column (100% dimethylsiloxane capillary; 30 m × 250 m × 0.25 m), a helium flow rate of 1.75 mL/min, and an injection port temperature of 250 °C. The sample was split at the column outlet between a flame ionization detector (HP7890A, Agilent Technologies, Santa Clara, CA) and a mass spectrometer (HP5975C). Electron impact mass spectra were collected using positive polarity and 70 eV.

5.2.5 Cell lysis and protein concentration determination

Cell pellet was resuspended in lysis buffer at pH 8.0 plus 100 µM PMSF, then sonicated on ice with Qsonica sonicator Q500 (Newton, CT) with microtip at amplitude 4.0 for 2 minutes with 2-seconds sonication and 3-seconds break intervals. After that, 2 µl of Benzonase® nuclease was added and incubated for 1 hour for degrading nucleic acid. The sample was centrifuged at 14,000 rpm at 4°C for 10 minutes. The supernatant from the cell lysis product was collected as cell extract for protein concentration determination and enzymatic reaction.

The protein concentration of the cell extract was determined by using Bradford protein assay reagent. The original Bradford reagent needed to be diluted with 4 parts of distilled and deionized water. Standard curve were established before any sample measurement. Standards concentration included 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of BSA. 100 µl of each standard and sample solution into 5 ml of diluted Bradford reagent, vortexed for 5 seconds and rested for 5 minutes before taking the measurement at 595 nm.

5.2.6 Lactate assay

To determine the concentration of L-lactate and D-lactate in cell extracts, oxidation of lactate by exogenous LDH was followed as the absorbance increase at A_{340} upon production of NADH (Fig 9). The assays were carried out in ~1 ml assay volume. It included 125 µl of 6 mM β-NAD^+, 350 µl of supernatant from culture and 500 µl of CAPS buffer (pH 9). To make sure the A_{340} signal was stable, the absorbance at 340 nm was monitored for 30 minutes prior to beginning each assay. The assay was started by adding 2 µl of either D-lactate dehydrogenase or L-lactate dehydrogenase, and the absorbance was recorded for 30 min. The difference between initial reading and final reading of A_{340} was divided by the time taken in minute for further calculation.

5.2.7 Lactate dehydrogenase assay

LDH catalyzes the reduction of pyruvate to form lactate in the presence of NADH. It also catalyzes the reverse reaction, the oxidation of lactate to form pyruvate in the presence of beta-nicotinamide adenine dinucleotide (β-NAD^+) as an electron acceptor (Figure 8). It is more convenient to assay LDH activity in the direction of lactate oxidation because it produces NADH that absorbs ultraviolet light at 340 nm. NAD^+

does not absorb at 340 nm. For these reasons, the increase of absorbance at 340 nm can be measured as an indication of the production of NADH either for either lactate level or LDH enzyme activity.

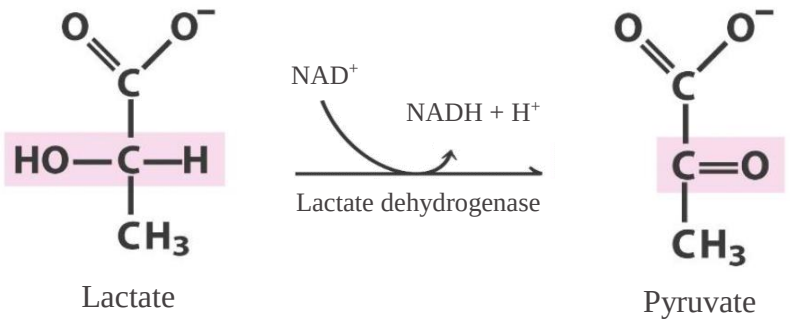


Figure 8. Enzymatic reaction of LDH converts lactate to pyruvate.

L-lactate dehydrogenase and D-lactate dehydrogenase activities in whole cell extracts of 1-phenylethanol grown propylbenzene adapted *Pp*F1 were assayed. In the ~1 ml reaction volume, 300 µl cell extract was added to 500 µl CAPS buffer, then 125 µl of 150 mM 6 mM β-NAD^+. The absorbance at 340 nm was monitored for 30 minutes prior to beginning each assay with the addition of lactic acid. The reading was started after 125ul of lactate was added. The difference between initial reading and final reading was recorded and will be divided by the time taken for further calculation.

5.3 Results and discussions

5.3.1 Another substrate giving rise to 1-phenylethanol

To determine if propylbenzene-adapted *Pp*F1 can grow on halo-alkylbenzene, 1-chloroethylbenzene was initially chosen and tested. In the growth study, 1-chloroethylbenzen strongly supported the growth for propylbenzene-adapted *Pp*F1. Surprisingly, growth on 1-chloroethylbenzene resulted in reducing of the pH of the culture medium to extreme low level. The cells grew higher than 2.0 at OD_{600} within 48 hours and the pH was as low as 1.8. The extreme low pH could be caused by the hydrolysis of chloride group from the 1-chloroethylbenzene and formed HCl (pKa = -3.0).

The stability of 1-chloroethylbenzene was further explored. It was found to be stable in solvents such as ethanol, methyl tert-butyl ether (MTBE) and ethyl acetate, but not in aqueous solution where it will spontaneously convert to 1-phenylethanol via nucleophilic substitution. Because 1-chloroethylbenzene is unstable in aqueous solution, it is hypothesized that the growth of the propylbenzene-adapted *Pp*F1 can actually be attributed to growth on 1-phenylethanol.

5.3.2 Characterization of 1-phenylethanol metabolism

Cell extracts were made from cells grown on 1-phenylethanol. The protein concentration of the cell extract was 0.8 mg/ml. In the lactate assay, 7.7 µmoles D-lactate was detected in culture media in 50 ml culture. In contrast, the L-lactate levels were below detection limit. Growth studies had already tested whether 0.2% (w/v) D-lactate and L-lactate would serve as the sole carbon in minimal medium for growing propylbenzene-adapted *Pp*F1 that had been exposed to 1-phenylethanol. The cells grew only using L-lactate but almost no growth with D-lactate. With supplying L-lactate as a sole carbon source, the cells grew from 0.1 to 2.0 within 24 hours in terms of OD_{600} signal. However, only 0.2 increments at OD_{600} was measured if the same amount of D-lactate was supplied.

In the lactate dehydrogenases activity measurement by using the cell extract, 6.6 $nmol \cdot min^{-1}mg^{-1}$ and 2.0 $nmol \cdot min^{-1}mg^{-1}$ in terms of specific activity for L-lactate dehydrogenase and D-lactate dehydrogenase, respectively, were detected. According to the result, L-lactate dehydrogenase was 3-fold higher than D-lactate dehydrogenase (Table 17). This could explain the difference in the extent of growth on L-lactate and D-lactate.

A caveat to these results is that the L-LDH and D-LDH are annotated as being potentially cytochrome-linked, ranther than NAD-linked, enzymes.

For this reason, more testing may be needed for verifying the difference between D-LDH and L-LDH in terms of enzyme activity and the growth.

It is also important to note that the propylbenzene-adapted *Pp*F1 could not use either D-lactate or L-lactate as a carbon source when the cells had not been exposed to 1-phenylethanol. This suggested induction of the lactic acid metabolism genes may only occur when lactic acid is generated intracellularly. This could happen because 1-phenylethanol should be able to partition into cells spontaneously whereas lactic acid woud need a dedicated transporter.

Based on analyzing the likely lactic acid gene region in the genome, PpuT_4600 is possibly the lactate operon regulator and Pput_4601 is annotated as L-lactate transport or L-lactate permease, Pput_4602 is annotated as L-lactate dehydrogenase and Pput_4603 is annotated as D-lactate dehydrogenase. The observation we mentioned above suggested that a putative lactic acid operon could be induced when 1-phenylethanol is supplied as a sole carbon source (Figure 9), but that the transporter is not functional.

In total, the data suggested that both (R)-1-phenylethanol and (S)-1-phenylethanol can be metabolized through TDO pathway and generate D-lactate and L-lactate (Figure 10).

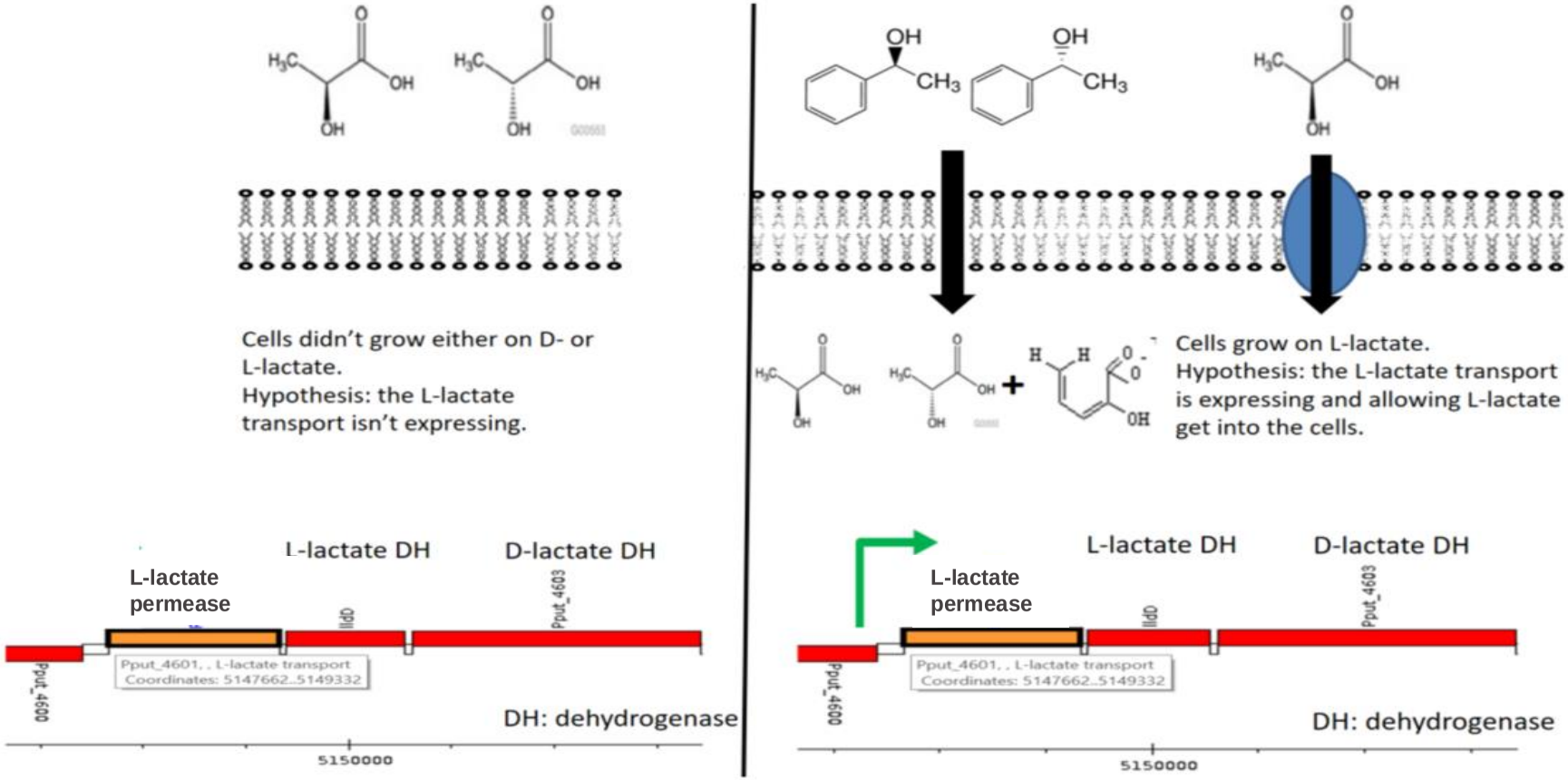
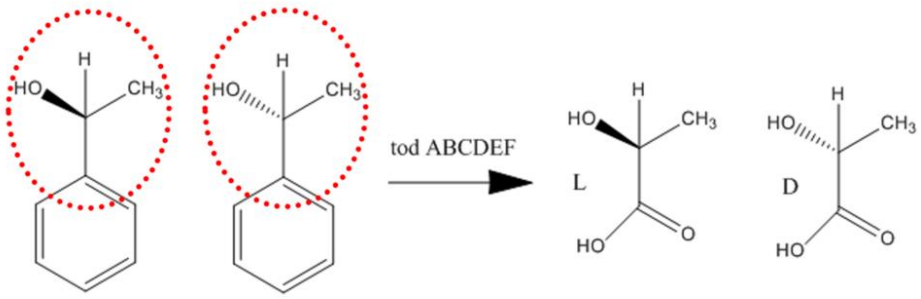


Figure 9. Hypothesis of lactic acid operon is induced while 1-phenylethanol is supplied.

Table 17. Lactate production from 1-phenylethanol and specific activity of lactate dehydrogenase.

	Growth (ΔOD_{600}) [a]		Conc.	Specific activity of LDH
	1-PE(–)	1-PE(+)	(μmoles)[b]	(nmol·min^{-1}mg^{-1})
D-lactate	<0.1	0.2	7.7	6.6
L-lactate	<0.1	2.0	ND	2.0

[a] The optical density at 600 nm for growth measurement before and after the cells exposed to 1-phenylethanol. 1-PE (–) indicates the cells before getting exposed to 1-phenylethanol; likewise, 1-PE (+) indicates the condition of cells that after exposed to 1-phenylethanol. [b] Lactate concentration in the supernatant from the culture media. ND: not determined.



Racemic 1-phenylethanol Lactic acids

Figure 9. Proposed reaction of lactic acid formation from 1-phenylethanol.

5.3.3 Investigations of 2-phenylacetamide metabolism

Using toluene dioxygenase knockout strains *Pp*F04 or *Pp*F106, and *Pp*F39/D no growth was observed on 2-phenylacetamide. This result was obtained three times and suggested that the growth using 2-phenylacetamide we observed in section 2.3.2 required toluene dioxygenase. To further investigate, we obtained phenylacetic acid pathway knockout strains RLF1 and RLF2, which have *paaF* and *paaI* gene mutated by using transposon insertion. In the growth study with these 2 strains, it showed that both RLF1 and RLF2 can grow on 2-phenylacetamide as a sole carbon source. These observations supported that 2-phenylacetamide is metabolized via TDO pathway (Figure 11).

However, there are caveats to the data above with additional experiments. First we did not detect the dihydrodiol intermediate product from the toluene dioxygenase reaction when using PpF39/D or pDTG601a, as we would have expected. Moreover, according to the result in the *meta*-fission intermediate screening assay (section 4.3) by using *E. coli* pDTG351, 2-phenylacetamide did not give 388 nm signal as the positive control toluene or other positive samples do (Figure 12). However, this could be due to the inability of *E. coli* to take up 2-phenylacetamide from the medium.

If the TDO pathway is operating to metabolize 2-phenylacetamide, malonamic acid would be expected to be produced by the TodF reaction (Figure 11). Malonamic acid or malonamide did not support growth as a sole carbon source but it did support growth as a sole nitrogen source to grow the propylbenzene-adapted *Pp*F1 when succinate or propylbenzene was supplied as a sole carbon source. Without supplying malonamic acid or malonamide, no growth was observed.

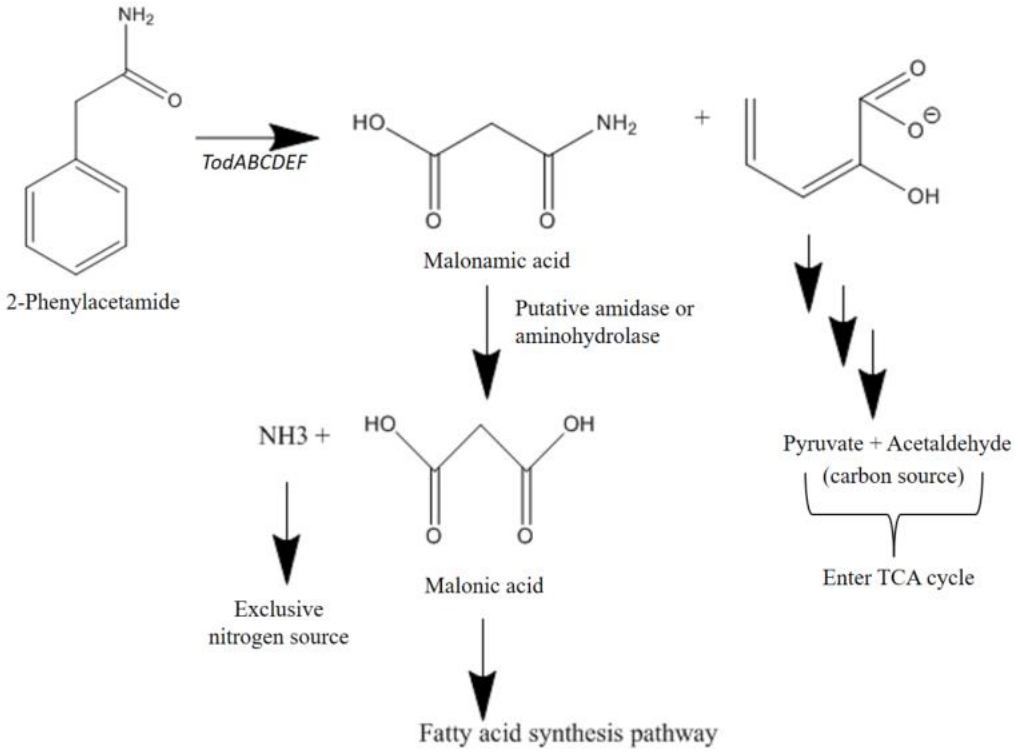


Figure 10. Putative 2-phenylacetamide metabolism pathway via TDO pathway.

Overall, our results in this section are most consistent with the growth of 2-phenylacetamide occurring via the TDO pathway. There is no direct evidence for the growth occurring via the phenylacetic acid pathway.

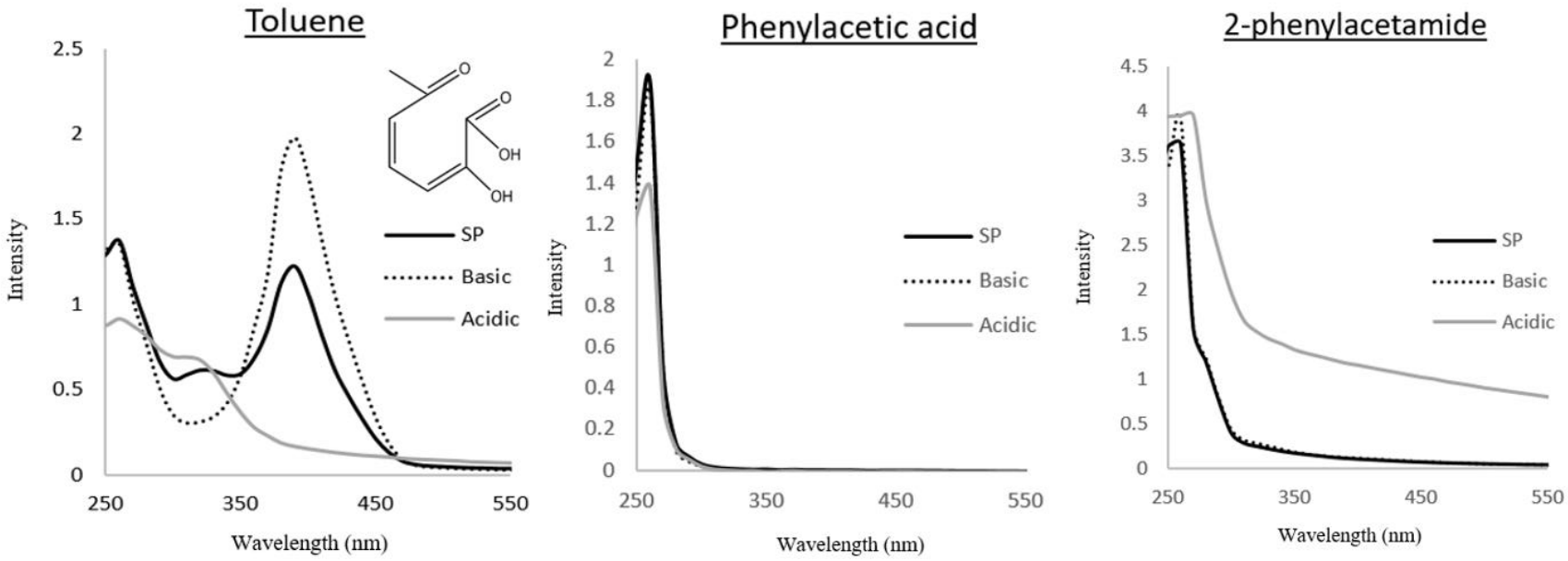


Figure 11. Comparison between spectrum profile of meta-fission intermediate screening assay between toluene (positive control), phenylacetic acid (negative control) and 2-phenylacetamide.

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CHAPTER 6

FUTURE WORK - WHOLE GENOME SEQUENCING

6.1 Introduction

Based on the discoveries from this study, we have learned that the propylbenzene-adapted *Pp*F1 has enhanced the ability to assimilate broader spectrum of aromatic compounds as growth substrate. It is unclear what has changed in terms of genetics. Based on Choi's work (Choi *et al*, 2003), it was considered that the mutations on *cym*R, encoding a repressor on the *p*-cymene operon could disrupt the inhibition by the repressor and consequently recruited a TodF equivalent ring-fission hydrolase CmtE to hydrolyze *meta*-fission intermediate that TodF could not react with. However, sequencing the *cym*R gene DNA from the propylbenzene-adapted *Pp*F1, we found the sequence to be identical to that published for the wild-type *Pp*F1 genome. For this reason, we would like to further explore the genomic difference between the wild-type *Pp*F1 and the propylbenzene-adapted *Pp*F1 using whole genome sequencing approach.

Among several platforms that are available on the market, we chose Oxford Nanopore Technologies (ONT). ONT's "strand sequencing" is a technique that passes intact DNA polymers through a protein called "pore" or "nanopore", sequencing in real time as the DNA translocates the pore.

This approach identifies nucleotides by measuring the changes in electrical conductivity when the DNA fragments passing through a pore that is immobilized on an electrically insulating membrane. Different level of current-flow signal from the background will be generated and recorded while the nucleotides are passing through the pore. This platform has been applied in many fields of biological works, such as resistome mapping (Helm et al, 2016), human gut microbiome metagenomics (Forsberg *et al*, 2012) and pathogen genome sequencing (Sommer et al, 2008).

It is possible that more than one gene has been mutated or that genomic DNA rearrangements have occurred that adapt *Pp*F1 and allow it to use propylbenzene and other aromatic compounds. In order not to consider artefacts that are not related to the adaption, a number of mutants will be subjected to whole genome sequencing. In addition, it is important to sequence our wild-type since the wild-type *Pp*F1 was sequenced ten years ago and we do not know if the sequence of the strain might have minor genetic changes from the one that used in our initial study.

In this section, the progress is still ongoing. We so far have done the growth, genomic DNA extraction, library preparation and sequencing. The sequence reads are being assembled and analyzed currently.

6.2 Materials and methods

6.2.1 Growth condition

Seven single colony from MSB plus propylbenzene (vapor) grown plate were picked and inoculated into 3 ml MSB and supplied with propylbenzene as sole carbon source. The cultures were incubated at 30°C with 250 rpm shaking for 48 hours. Cells were spun down in 15-ml falcon tube while the optical density at 600 nm reached 2.0. The supernatant was discarded and cell pellets were retained for genomic DNA extraction.

6.2.2 Genomic DNA preparation

Cell pellets are resuspended individually in 50 µl TE buffer (10 mM Tris-HCl, pH 8.0) by vortex agitation for 30 seconds. Then add 2 ml lysis buffer (10 mM Tris-HCl, pH 8.0; 25 mM EDTA; 20 µg/ml RNase A) and 20 ul lysozyme (from freshly prepared 100 mg/ml stock in TE buffer) was added. The suspension was vortexed at full speed for 5 seconds. After that, it was incubated at 37°C for 30 minutes. Then add 70 µl of 15% SDS to final concentration of 0.5% (w/v), incubate the tubes at 37°C for 1 hour. For denaturing proteins, 50 µl Qiagen proteinase K (stock concentration 20 mg/ml) was added and the samples were incubated at 50°C for 3 hours. Mix by slowly rotating end-over-end 10 times after 1 and 2 hours. After that, add

an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and placed on a rotator vertically for circular rotation at 40 rpm for 10 minutes. Then, it was centrifuged at 4500 rpm for 10 minutes. The aqueous phase was pipetted into a clean 15-ml falcon tube.

The following steps involved the DNA precipitation and clean-up. First, 200 µl volume 3.0 M sodium acetate (pH 5.2) was added to each sample, followed by adding of 2 volumes ice-cold 100% ethanol. The samples were inversely rotated end-over-end for 10 times and placed on ice. At this point, we used a sterile glass hook to spool out the precipitated genomic DNA and submerge the hook in a clean 1.5-ml Eppendorf tube containing 70% ethanol. The opaque DNA was carefully taken off the rod into a clean, dry, 1.5-ml Eppendorf Lobind microtube.

In order to obtain a highly quality genomic DNA, more clean-up steps were applied. For each sample, 1 ml ice-cold 70% ethanol was added, followed by centrifugation with 10,000 x g for 5 minutes at 4°C. After removing ethanol from the second wash, the samples were allowed to evaporate the residual ethanol by placing the tubes in a 40°C heat block for 10 minutes. Finally, 50 µl nuclease free water and 0.5 µl RNAse A were added. The samples were incubated at 37°C for 30 mins, followed by incubation at 4°C overnight for full rehydration.

In the next day, DNA concentration was measured with Qubit fluorescent meter in the broad range double strand DNA mode. In addition, we also measured 260:280 and 260:230 ratios using Nanodrop One UV/Vis spectrophotometer (Thermo-Scientific, Waltham, MA).

6.2.3 Library preparation

The procedure of making library is following the standard protocol of one-dimension barcode ligation for genomic DNA that released by Oxford Nanopore Technology (2016, Version 1.03)

6.2.4 Metichor cloud-based or local-based base calling

The MinKNOW software (version 1.0.5) was used to control the sequencing process and the read files were uploaded to the cloud based Metrichor EPI2ME platform for base calling. Base called reads were downloaded for further processing and assembly.

6.3 Reference

Choi E.N., Cho M.C., Kim Y., Kim C.K. & Lee K. (2003) Expansion of growth substrate range in *Pseudomonas putida* F1 by mutations in both *cym*R and *tod*S, which recruit a ring-fission hydrolase CmtE and induce the *tod* catabolic operon, respectively. *Microbiology* 149, 795-805.

Helm E. V., Imamovic L., Ellabaan M. H., Schaik W., Koza A., and Sommer M. O. A. (2016) Rapid resistome mapping using nanopore sequencing. *Nucleic Acids Research*, 1–8.

Pehrsson, E.C., Tsukayama, P., Patel, S., Mej'ıa-Bautista, M., Sosa-Soto, G., Navarrete, K.M., Calderon, M., Cabrera, L., Hoyos-Arango, W., Bertoli, M.T. *et al.* (2016) Interconnected microbiomes and resistomes in low-income human habitats. *Nature*, 533, 212–216.

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CHAPTER 7

CONCLUSION

In this study, we confirmed that an adapted *Pseudomonas putida* F1 that grows on additional aromatic is readily obtained. Wild-type *Pp*F1 grows on a narrow spectrum of aromatic hydrocarbons as a growth substrate, such as benzene, toluene, ethylbenzene, *p*-cymene and phenylacetic acid. However, *Pp*F1 can be domesticated to grow on biphenyl, *n*-propylbenzene, isopropylbenzene and *n*-butylbenzene to generate a strain that grows on a broader range of aromatic hydrocarbons. There are several aromatics catabolism pathway in *Pp*F1, such as benzoate dioxygenase, phenylacetyl-CoA epoxidase, p-cymene-monooxygenase, and toluene dioxygenase-mediated pathways. Among them, toluene dioxygenase is believed to be the one that is highly flexible, supporting growth on various aromatic compounds in this study. There is a possibility for interplay or cross-interaction between the aromatic catabolism pathways to broaden the growth range.

In the growth study of propylbenzene-adapted *Pp*F1, this study used dozens of aromatic compounds. Besides the known growth aromatic growth substrates, we further identified 13 novel growth substrates, which include

allylbenzene, 4-phenyl-1-butene, biphenyl, cyclohexylbenzene, cyclopropylbenzene, diphenylmethane, 2-chloroethylbenzene, 1-phenylethanol, 2-phenylacetamide, phenyl formate, phenyl acetate, benzylamine and 3-phenylpropionaldehyde. In contrast, propylbenzene-adapted *Pp*F1 does not grown on 1-phenyl-3-pyrazolidone, 1,3-benzodioxole, 2-phenyl-2-oxazoline, 2-phenylpyrrolidine, benzamide, phenyl carbamate, phenylurea, phenylboronic acid, *n*-butyl phenyl ether, acetophenone, benzyl alcohol, cinnamyl alcohol, *p*-cresol, anisole, 3-methylanisole, thioanisole, diphenyl sulfide and diphenyl ether. All positive-growth results were at least duplicated to confirm the results.

Although we could not detect all possible organic acids from each aromatic precursor in the culture medium to confirm these growth substrates, the results are consistent with the metabolism via the TDO pathway. However, we tested every one of them to further validate the possibility. Additionally, we also performed *meta*-fission intermediate screening assay to examine the existence of the unique *meta*-fission intermediate product which is produced via *E. coli* containing TodABCDE. As we expected, most of the results were matched to each other from these three approaches, which strongly indicates that they were metabolized via TDO pathway.

There are some data that do not perfectly match in these three approaches, such as 4-pentenoic acid – a putative TodF product in the TDO pathway of 4-phenyl-1-butene, which did not support growth as a sole carbon source. However, 4-phenyl-1-butene showed an obvious 388 nm spectrum in the *meta*-fission intermediate assay in the high pH condition, which suggested that it is a substrate for TodABCDE. It is possible that the growth by supplying 4-phenyl-1-butene was supported by another TodF product – *cis*-2-hydroxypenta-2,4-dienoate, which eventually will be converted into pyruvate and acetaldehyde. If that is the case, the 4-pentenoic acid is supposed to accumulate and does not have toxicity to the cells.

The lactic acid operon hypothesis may be caused by artifact, although the no growth result was triplicated to confirm that *Pp*F1 cannot use both enantiomers of lactic acids as a sole carbon source, however, the 1-phenylethanol grown propylbenzene-adapted *Pp*F1 could grow on L-lactic acid but almost no growth on D-lactic acid.

CHAPTER 8

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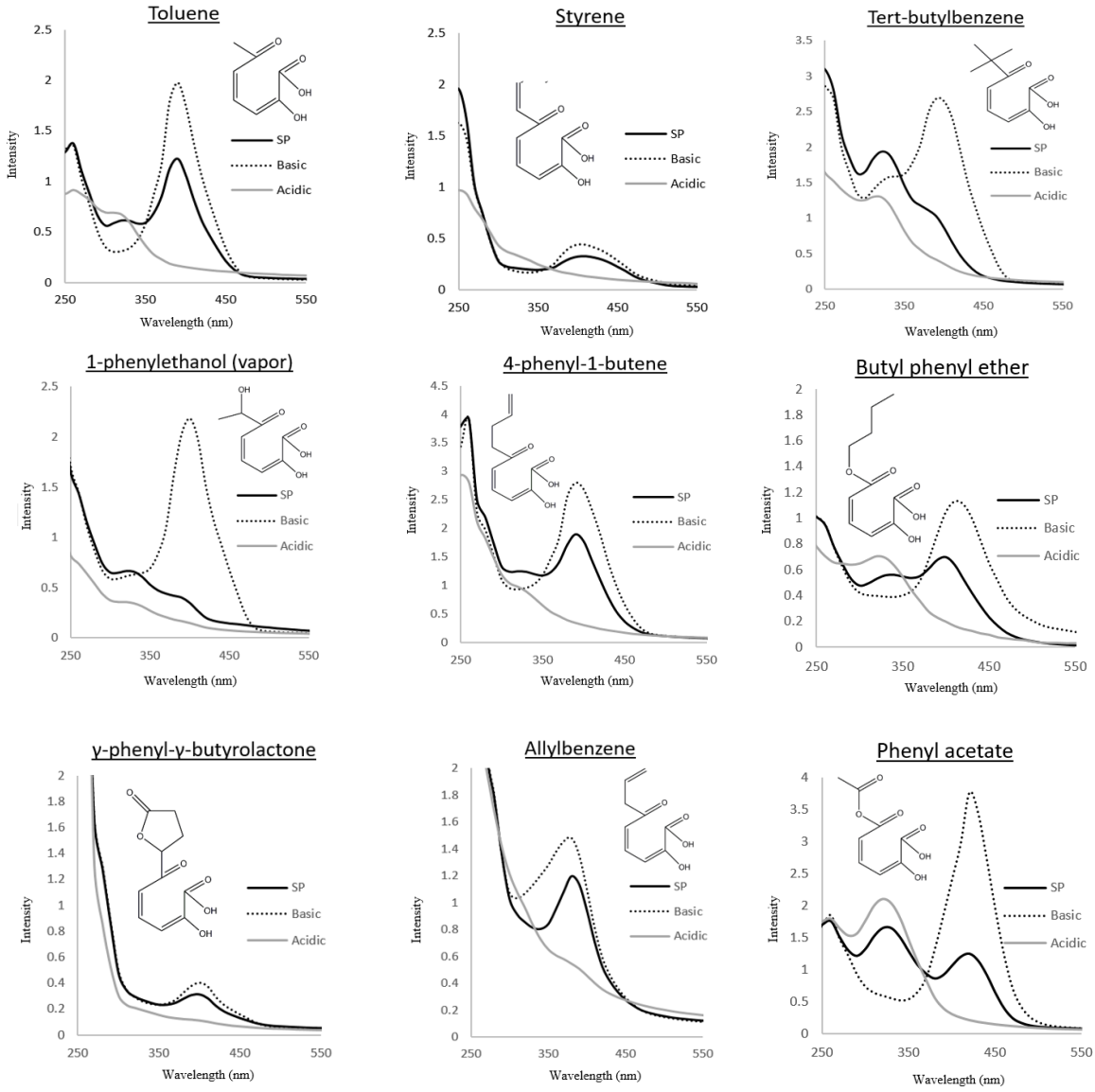
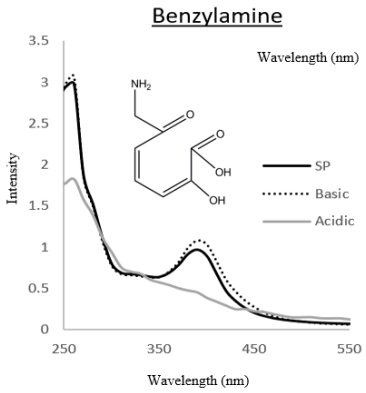
CHAPTER 9

SUPPLEMENTARY DATA / APPENDIX

9.1 Supplementary data I

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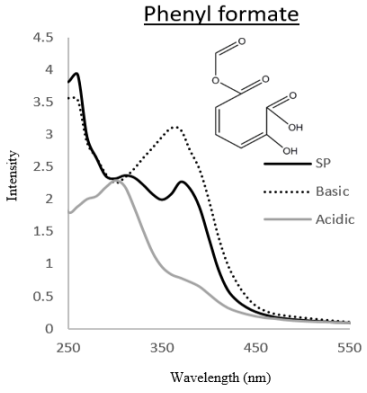
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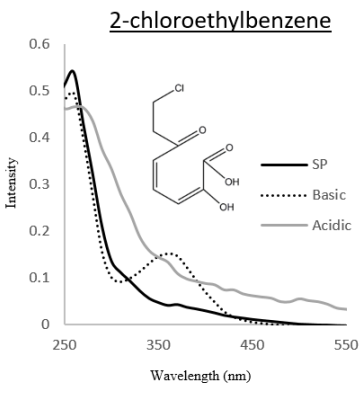
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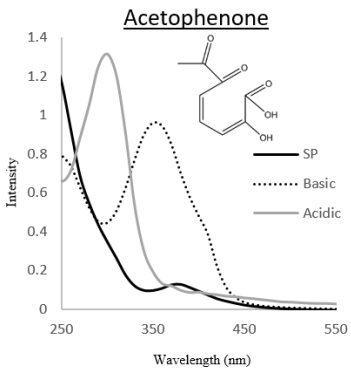
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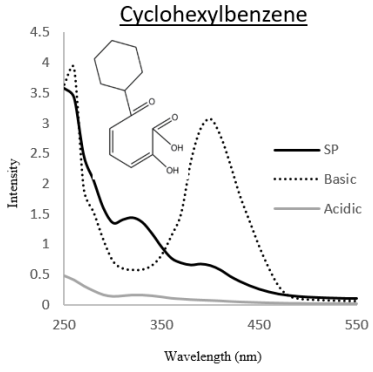
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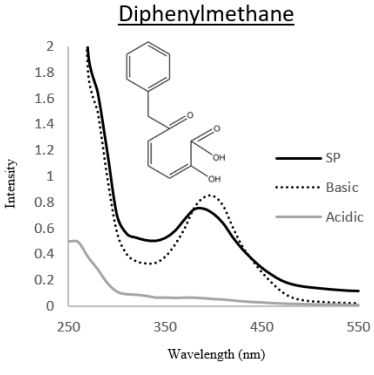
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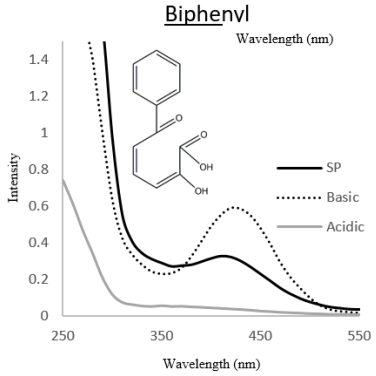
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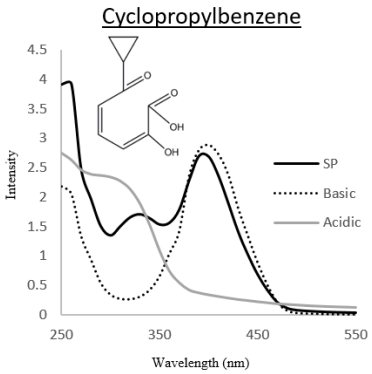
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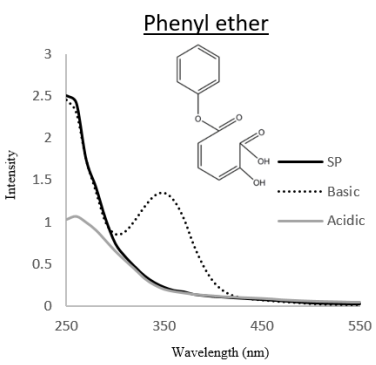
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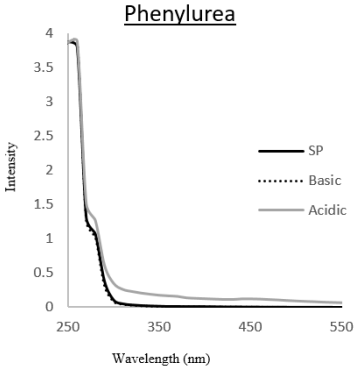
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Basic
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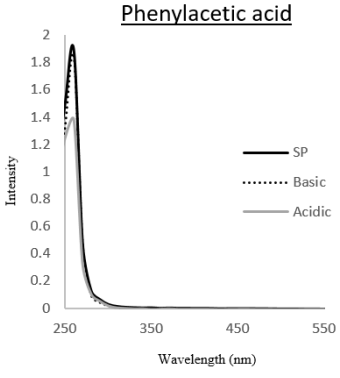
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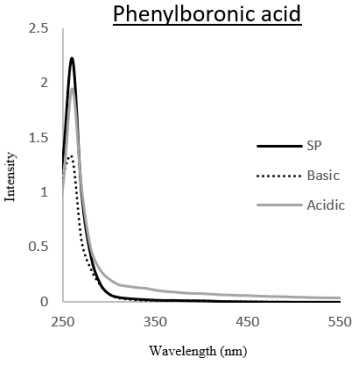
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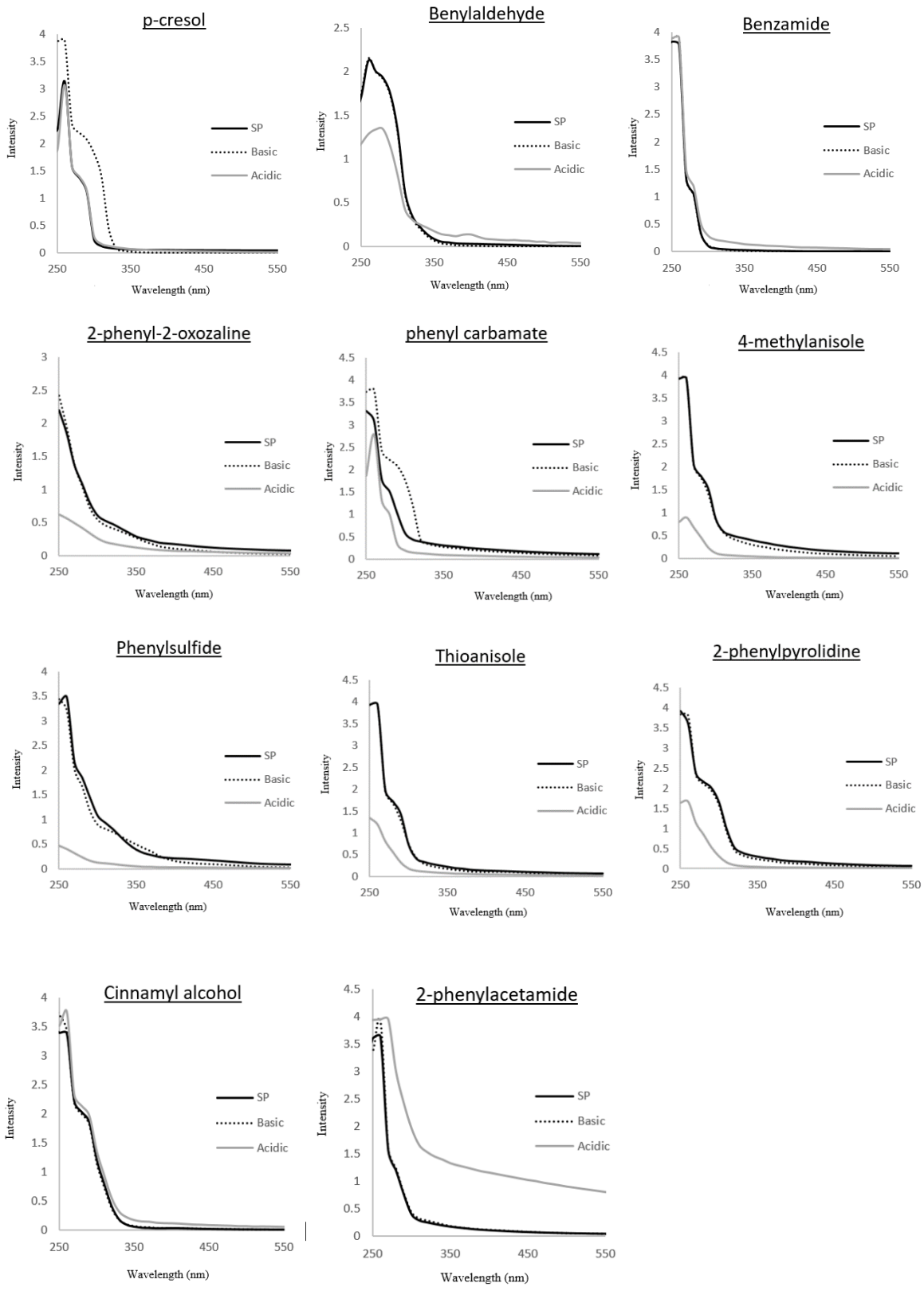


Appendix I. *Meta*-fission intermediate pattern spectrum.

9.2 Appendix I – Minimal salt basal medium recipe

Ingredients of minimal salt base medium (1 liter):

1.00 g of NH_4Cl

3.49 g of $Na_2HPO_4 \cdot 2H_2O$

2.77 g of KH_2PO_4

20 mL of Hunter's vitamin-free mineral base with each liter of MSB

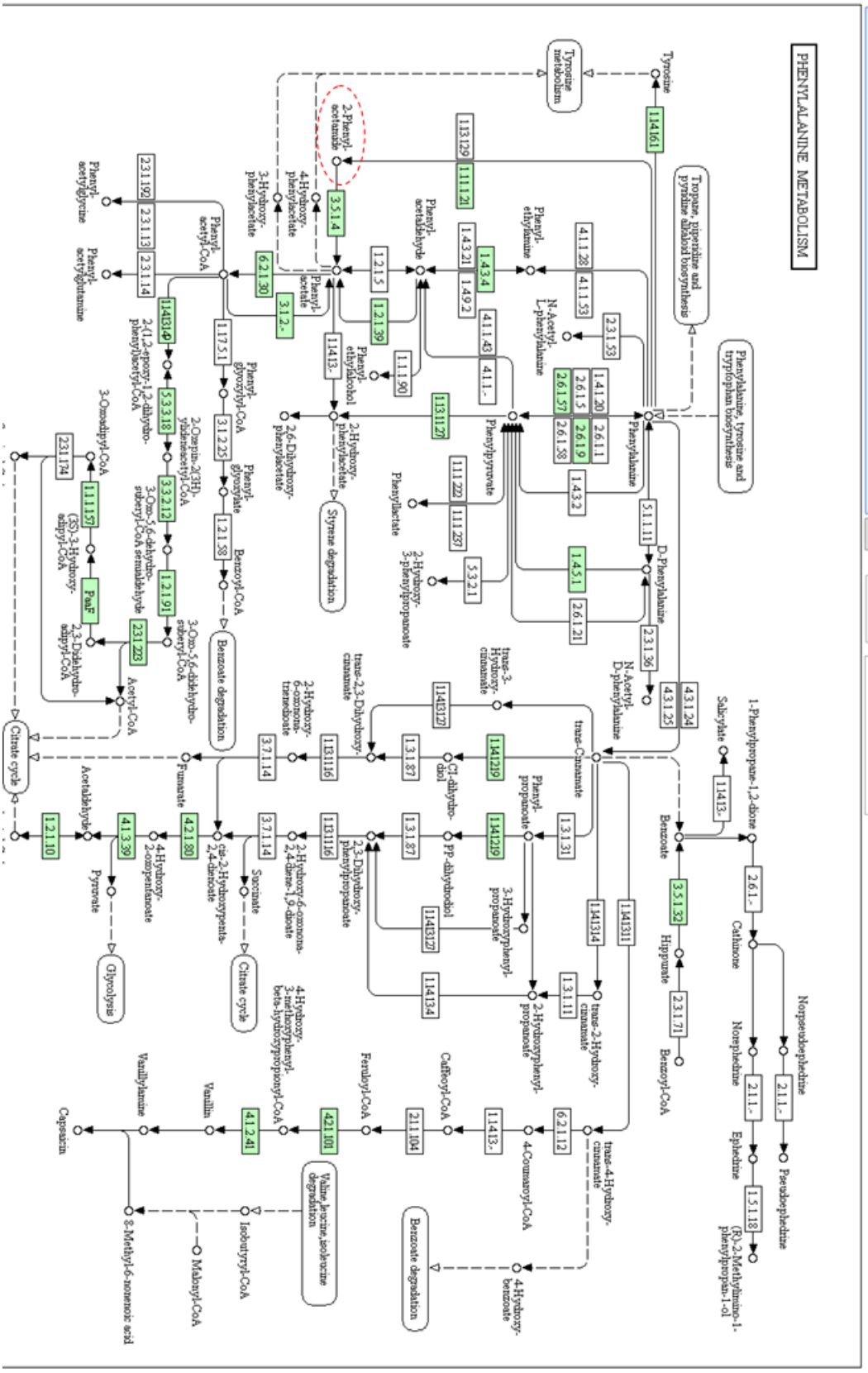
980 mL of distilled and deionized water, adjusted the pH to 6.8

Hutner's vitamin-free mineral base contains the following (per liter):
10 g of nitrilotriacetic acid (neutralize with 6.00 g of KOH)
14.5 g of $MgSO_4 \cdot 7H_2O$
3.33 g of $CaCl_2 \cdot 2H_2O$
9.74 mg of $(NH_4)_6Mo_7O_2 \cdot 4H_2O$
99 mg of $FeSO_4 \cdot 7H_2O$
50 mL of the Metals 44 solution

The composition of Metals 44 (per 100 mL) was as following:
318 mg of Na_2 EDTA$\cdot 2H_2O$
1.10 g of $ZnSO_4,7H_2O$
914 mg of $FeSO4,7H_2O$
154 mg of $MnSO_4,H_2O$
39.2 mg of $CuSO_4,5H_2O$
24 mg of $CoSO_4 \cdot 7H_2O$
17.7 mg of $Na_2B_4O_7 \cdot 10H_2O$ and was neutralized with H_2SO_4

The vitamin solution (per 100 mL) was prepared by mixing:
0.50 mg of biotin
50 mg of nicotinic acid
25 mg of thiamine hydrochloride

9.3 Appendix II – Phenylalanine metabolism pathway on KEGG



End of thesis